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ZUSAMMENFASSUNG

Die Klasse der Bedecktsamer besticht mit einer riesigen Blüten- und Artenvielfalt, und kann auf der ganzen Welt gefunden werden. Die Suche nach den Ursachen für diese pflanzliche Vielfalt ist eines der wichtigsten Ziele der Evolutionsbiologie und treibt seit ihrem Anfang die Forschung der Evolutionsbiologen an. Ein Schlüssel für das Verständnis dieser Vielfalt liegt in den vielfältigen Wechselwirkungen zwischen den Bedecktsamern und ihren Bestäubern. Denn diese nehmen durch das Ermöglichen der sexuellen Fortpflanzung in Pflanzen eine wichtige Doppelfunktion ein. Einerseits können Bestäuber auf Blütenmerkmale Selektion ausüben, anderseits können sie als Fortpflanzungsbarrieren agieren. Eine grundlegende Hypothese der Evolutionsbiologie besagt daher, dass die pflanzenseitige Anpassung an unterschiedliche Bestäuber einer der Hauptmechanismen ist, welcher die Blüten- und Artenvielfalt antreibt.

Das Ziel der vorliegenden Arbeit ist die eingehende Untersuchung des Anpassungsprozesses von Bedecktsamern mit spezialisierten und generalisierten Bestäubungssystemen an die Bestäuber. Der Fokus liegt auf der durch die Bestäuber vermittelten Selektion, der Evolution von Blütenmerkmalen sowie der Bestäuberisolation. Insbesondere wurden die Auswirkungen von Änderungen in der Bestäuberzusammensetzung auf die Entwicklung von Blütenmerkmalen (wie Geruch, Farbe und Morphologie), und Paarungssystemen durch experimentelle Evolution quantifiziert. Des Weiteren wurde die Bedeutung von effizienten Bestäubern auf die Blütenselektion und -evolution in gemischten Bestäuberumwelten untersucht. Durch die Untersuchung von Fortpflanzungsbarrieren zwischen zwei eng verwandten, sexualtäuschenden Orchideen wurde zudem die Anpassung an Bestäuber sowie die Stärke und Bedeutung von Bestäuberisolation im Prozess der Artbildung bewertet. Zur Klärung dieser Forschungsfragen wurden insgesamt drei Studien mit Bedecktsamern durchgeführt, welche in den drei Kapiteln dieser Arbeit beschrieben sind.

In Kapitel I wurde durch experimentelle Evolution über neun Generationen die von Bestäubern vermittelte Selektion und die Evolution von Blütenmerkmalen in schnell wachsenden Rübsen (*Brassica rapa*) untersucht. Diese Pflanzen wurden getrennt zwei unterschiedlichen Bestäuberumgebungen ausgesetzt; und zwar einer Umgebung von Hummeln (*Bombus terrestris*) sowie Schwebefliegen (*Episyrphus balteatus*). Die

Ergebnisse zeigen, dass sich die beiden Bestäuber in der Selektion auf Blütenmerkmale unterschieden; so selektionierten nur die Hummeln über den Blütenduft. Als Reaktion auf die unterschiedlichen Bestäuber- und damit Selektionsumgebungen konnte bei mehreren Blütenmerkmalen eine evolutionäre Veränderungen beobachtet werden. Zusätzlich konnten im Verlauf der Zeit durch die Zunahme von autonomer Selbstbestäubung in der Schwebefliegenumgebung, welche eine tiefe Bestäubungseffizienz zeigten, Veränderungen in der pflanzlichen Fortpflanzungsstrategie beobachtet werden.

In Kapitel II kommt der gleiche Ansatz wie in Kapitel I zur Anwendung, jedoch waren diesmal beide Bestäuber gleichzeitig vorhanden. Dies erlaubte die Beantwortung der Frage, ob der effizientere Bestäuber eine ausgeprägtere Selektion ausübt und die Evolution von Blütenmerkmalen stärker bestimmt. Diese Experimente zeigen, dass die Präferenzen des effizienteren Bestäuber nicht zu einer stärkeren Selektion für diese Blütenmerkmale führen. Darüber hinaus hat sich gezeigt, dass wenig effiziente Bestäuber die Selektion des effizienteren Bestäubers beeinflussen und ändern können.

In Kapitel III wurden schliesslich die Stärke der einzelnen Fortpflanzungsbarrieren zwischen zwei mutmasslichen Schwesterarten von sexualtäuschende Orchideen (*Ophrys insectifera* und *Ophrys aymoninii*) quantifiziert. Hierbei konnte festgestellt werden, dass beide Orchideen an Ihre Bestäuber angepasst sind und die Bestäuberisolation durch die Attraktion von spezifischen Bestäubern mittels Blütenduft als hauptsächliche Fortpflanzungsbarriere zwischen den beiden Arten in Sympatrie agiert. Darüber hinaus zeigten beide Orchideenarten eine starke Überlappung in ihren Mykorrhizapartnern, was darauf hindeutet, dass diese wenig zur Fortpflanzungsisolation beitragen.

Insgesamt erlaubt diese Arbeit einen detaillierten Einblick in den Prozess der Anpassung von Bedecktsamern an ihre Bestäuber. Sie zeigt auf, wie Unterschiede in der Bestäuberumgebung von Pflanzen mit generalisierten Bestäubungssystemen innert kurzer Zeit die Divergenz von Blütenmerkmalen (besonders beim Blütenduft) und Veränderungen in der Paarungsstrategie vorantreiben können. Darüber hinaus konnte festgestellt werden, dass in gemischten Bestäuberumgebungen der effizientere Bestäuber nicht zwangsweise die Selektion bestimmt und dass auch wenig effiziente Bestäuber einen messbaren Einfluss auf die Selektion ausüben. Die Auswertung der Experimente führen zum Schluss, dass in spezialisierten Bestäubungssystemen die Bestäuber den entscheidenden Faktor in der Evolution von Blütenmerkmalen unter Fortpflanzungsisolation spielen. Diese Arbeit unterstreicht, dass Bestäuber eine

wichtige Rolle in der Divergenz von Blütenmerkmale sowie in der Artbildung von Pflanzen spielen. Mit der vorliegenden Arbeit konnte schliesslich auch die grosse Bedeutung von experimenteller Evolution für das Studium von Anpassungen von Pflanzen an Bestäuber nachgewiesen werden, weshalb dies in der Forschung von Pflanzen-Bestäuber-Beziehungen noch ein grosses Potenzial aufweist.

SYNOPSIS

The angiosperms captivate us with a huge floral and species diversity found all around the world. To find the causes and drivers of this diversity is one of the major aims in evolutionary biology and has since its beginning fueled the research of evolutionary biologist. One key for understanding this diversity lies in the manifold interactions between plants and pollinators. The reason is found in the dual-function pollinators have on plants by enabling sexual reproduction and as a consequence can impose selection on floral traits as well as causing reproductive isolation. It is therefore a major hypothesis in evolutionary biology that adaptions to different pollinators are one of the major mechanisms that drive floral diversity and plant speciation.

The aim of this thesis is the in-depth study of the process of plant adaptations to pollinators in generalized and specialized plant systems. Focus was laid on pollinator-mediated selection, floral trait evolution and floral isolation. Specifically, the effects of pollinator shifts on floral trait (i.e. scent, color, morphology) evolution, mating-systems and reproductive isolation were quantified in an experimental evolution approach in a generalized plant. Furthermore the importance of efficient vectors in mixed pollinator environments on selection and floral trait evolution were examined. Additionally, through the study of isolation barriers between closely related sexually deceptive orchids pollinator adaptation as well as the strength and importance of floral isolation in the process of speciation was evaluated. In total three studies were conducted and are detailed in the three chapters of this thesis.

In Chapter I, in an experimental evolution approach, pollinator-mediated selection and floral trait evolution was quantified over nine generation in the rapid-cycling *Brassica rapa*, which were exposed to two different pollinator environment separately, namely a bumblebee (*Bombus terrestris*) and a hoverfly (*Episyrphus balteatus*) environment. The results showed that the two pollinators differed in the selection they imposed on the plant traits, specifically only bumblebees showed strong selection on floral scent compounds. Additionally multiple floral traits have undergone evolutionary changes in response to the pollinator environments and selection therein. Moreover, changes in mating strategies were also observed over time as autonomous-selfing became more prevalent in the low-efficient hoverfly treatment.

In Chapter II, we used an identical approach as in Chapter I but with both

pollinators simultaneously present to measure if the more efficient pollinator imposes the strongest selection and drives floral evolution. Our results showed that the preferences of the more efficient pollinator did not transfer into stronger selection for these floral traits. Moreover it shows that low-efficient pollinators may influence and alter the selection imposed by more efficient pollinators.

In Chapter III, the strength of individual reproductive barriers was quantified between a pair of putative sister-species, namely the sexually deceptive orchids *Ophrys insectifera* and *Ophrys aymoninii*. The results show that both orchids are adapted to their pollinators and that floral isolation, specifically the attraction of specific male insects trough floral scent, acts as the main reproductive barrier between the two species in sympatry. Moreover, both orchids were found to overlap in mycorrhizal fungi partners, suggesting that fungal partners most likely do not contribute to reproductive isolation.

Overall this study offers a detailed insight into the process of plant adaptation to pollinators. This thesis highlights how shifts in pollinators can mediate floral trait divergence, especially floral scent, and shape plant-mating strategies in generalized plants within a short time. Additionally, it also shows that in mixed pollinator environments, higher pollination efficiency does not necessarily transfer into stronger selection and that low-efficient pollinators can also influence net selection. Moreover, in specialized pollination systems pollinators have been shown to act as major driver of floral adaptions and reproductive barriers. This thesis highlights that pollinators play an important role in floral divergence and also plant speciation. Finally, this study shows the strength and significance that experimental evolution experiments can have on the study of plant adaptation to pollinators and should be therefore applied more often in plant-pollinator research.

GENERAL INTRODUCTION

Floral diversity and the origin of pollination biology

Walking along a meadow, in mountain grasslands or tropical rainforests one can only be amazed by the stunning diversity of flowering plants that can be found all around the globe. Current estimations assume that there around 352'000 flowering plants worldwide, but some estimates go as high as 400'000 species (Paton et al. 2008; Pimm and Joppa 2015). The flowering plants show a huge variety in floral colors, shapes and perfumes from the miniscule flowers of the orchid *Platystele* to the gigantic *Amorphophallus* with its carrion-like odor. Also within plant species a wide variability of floral traits can be observed. More importantly, on the flowers also a huge diversity of animal visitors can be found, spanning from insects over birds to mammals. Such observations will ultimately raise questions about the origin and function of these diverse flowers and the relation they have with their animal visitors. Indeed, these questions are one of the central topics in evolutionary biology and have opened up a huge field of research.

The origin of pollination biology dates back into the 18th century from the work of C.K. Sprengel. In his cornerstone book "*Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen*" from 1793 (Fig 1), Sprengel acquired a deep insight into function of flowers and their relations to pollinator trough the study of nearly 500 plants in his vicinity (Sprengel 1793). He demonstrated that plants need insects for pollination and setting seeds while insect visitors feed on the nectar (called "Saft" by Sprengel) often offered by plants. He was the first to extensively show the importance insects have in the reproduction of flowering plants by transport of pollen between flowers but also to highlight the diversity of pollination systems. In his work, his view was that the colorful flowers act as an attractant for the insects and that not each color is identical attractive to insects as others. Moreover he indicated that different flowers might be destined for different insects, which could be regarded as floral adaptations to certain pollinators. Unfortunately, at the time his achievements have not received the recognition they deserved. It was not until Darwin, who gave his work the attention and appreciation it deserved (Darwin 1877). He not only often approved of Sprengel's conclusions but also confirmed the important role that insects play in plant pollination.

He saw in pollination a mean of avoidance of selfing, which increases the variation of plant traits on which selection may act. Furthermore, Darwin established in his book "*On the various contrivances by which orchids are fertilised by insects*" that a great variety of the forms found in orchids had arisen from natural selection to facilitate pollination and also hypothesized that pollinators may have played an important role in the evolution of flowering plants. Thus the foundation was laid for the future research in the field of pollination biology.

Fig 1: Frontispiece of C.K. Sprengels book "*Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen".*

The basic principles of plant-pollinator interactions

Since the work of Sprengel and Darwin the knowledge in the field of plant-pollinator interactions has risen significantly. Today, it is assumed that over 85 % of all angiosperms are pollinated by animals, which highlights how strong plants rely on them (Ollerton et al. 2011). Plants use these animal pollinators as pollen vector for sexual reproduction. Compared to wind pollination, animal pollinated plants are thought to benefit from such a relation by more specific pollen flow in lower population densities, through higher outcrossing rates and reduced pollen production (Pellmyr 2002). The pollinators on the other hand do not provide this service for free. Pollinators visit flowers in search for food in form of readily accessible high-quality nectar and pollen, but also for brood-rearing and sexual partners (Faergi and van der Pijl 1979; Pellmyr 2002). However, not all plants offer a reward, which was also observed and described by Sprengel as "Scheinsaftblüten", but rather deceive the pollinators in pretending to provide a reward or in extreme cases sexual partners (Schiestl et al. 1999; Schiestl 2005; Jersakova et al. 2006). Such deception has been found to be an especially widespread mode of pollination in orchids (Cozzolino and Widmer 2005; Schiestl 2005; Jersakova et al. 2006; Schiestl and Schluter 2009; Gaskett 2011). On the other hand, highly mutualistic interaction as found in the fig/fig-wasp represent the opposite side of the plant-pollinator relationship (Machado et al. 2005).

Although plants offer rewards (or not) to their pollinators, these rewards need to be advertised to the animal visitors for increasing the efficiency in pollen transfer (Faergi and van der Pijl 1979; Harder et al. 2001; Pellmyr 2002). To attract these pollinators, plants have evolved a staggering array of advertisements in form of floral signals like shape, scent or color (Pellmyr 2002; Raguso 2008; Schiestl 2010; Schiestl and Johnson 2013). The pollinators have been shown to learn these floral signals and associate them with rewards, or in case of deception to avoid them, allowing them to gather high quality resources more efficiently, a process described as "floral constancy" (Goulson 1999; Ayasse et al. 2000; Wong and Schiestl 2002; Gaskett 2011; Knauer and Schiestl 2015). In combination with pollen limitation, the pollinators can therefore impose selection on floral traits they prefer (Waser and Campbell 2004; Johnson 2006). Overall, it has been shown that floral traits are adaptations towards pollinators (Harder and Johnson 2009). Moreover, support for the importance of pollinators in shaping floral traits can be found in the convergent evolution of floral traits in different plant

families, termed pollination syndrome (Fenster et al. 2004). There is also increasing phylogenetic support that pollinators acted as a main driver of diversification in flowering plants (Dodd et al. 1999; Kay and Sargent 2009; Van der Niet and Johnson 2012; Forest et al. 2014). Today it is commonly acknowledged that pollinators played an essential role in floral evolution and plant diversification.

Pollinators as driver of floral diversity

The conceptual model

The conceptual model for pollinator-driven diversification in flowering plants, originated from the work of Grant & Grant and Stebbins (Grant and Grant 1965; Stebbins 1970) and has been developed and promoted as the Grant-Stebbins model by Johnson (Johnson 2006, 2010). The underlying principle of the model is that due to different pollinator environments with different selection regimes, plants are under divergent selection and adapt to their local most effective pollinators, therefore causing floral traits to diverge among populations. With time these divergences may eventually become large enough to contribute to reproductive isolation and ultimately speciation.

Pollinator-mediated selection and adaptation to pollinator ecotypes

As pollinators fulfill an essential role in plant reproduction, they can have direct effects on plant fitness and therefore impose strong selection on floral traits (Waser 1998). Such selection on floral traits has been shown to be different between pollinators, allowing plant traits to diverge under these different selection regimes (e.g. (Galen et al. 1987; Campbell et al. 1997; Medel et al. 2003; Gomez et al. 2008; Gomez et al. 2009; Gross et al. 2016). The variation in selection arises from differences in the pollinator preferences on floral traits as the pollinators differ in morphology, food requirements and the way floral signals are perceived by them (Galen et al. 1987; Lunau 2000; Vereecken et al. 2010; Schiestl and Johnson 2013). In general, phenotypic selection studies have been performed predominantly on morphological and visual traits (Kingsolver et al. 2001; Gomez et al. 2009; Sletvold and Agren 2010; Sahli and Conner 2011; Sletvold et al. 2016). For a long time neglected, mainly due to technical limitation, selection on floral scent has shown to become a crucial part in plant-insect interactions

(Schiestl et al. 2011; Ehrlén et al. 2012; Parachnowitsch et al. 2012; Gross et al. 2016). Further studies incorporating olfactory floral signals, will thus help us to complement the understanding we have on floral trait evolution. Additionally, while the mechanisms driving among-population floral divergence (divergent/directional selection) are prevalent in studies and often the focus of research, it has been hypothesized that such trait divergence may also occur within populations trough disruptive selection (Waser and Campbell 2004). Such processes are thought to occur especially in sexually deceptive orchids, which have a highly specialized pollination systems and where mutants might attract trough novel floral signals different pollinators (Johnson 2006; Vereecken et al. 2010; Xu et al. 2012b).

As pollinators have been found to differ in their distribution spatially and temporally, they create therefore a geographic mosaic of pollinator abundances (Grant and Grant 1965; Williams et al. 2001; Herrera et al. 2006; Johnson 2006). Such pollinator mosaics or shifts in the pollinator ecotype can be consequences of abiotic (e.g. temperature, altitude) and biotic (e.g. availability of flowering plants) factors that can limit the range and abundance of pollinators (Parmesan et al. 1999; Geber and Moeller 2006; Kerr et al. 2015). These variable pollinator environments and differences in the selection regime may thus promote trait divergence as plants adapt to the local pollinator environments (Grant and Grant 1965; Stebbins 1970). Phylogenetic studies show that pollinator-shifts and angiosperm-diversification are to some extend linked with each other (Van der Niet and Johnson 2012). Great examples of such adaptations to local pollinator environments are known form long-proboscid flies in the *Disa draconis* complex (Johnson and Steiner 1997) or from plants pollinated by the long-proboscid fly *Prosoeca longipennis*, which has a geographic variation in tongue length (Newman et al. 2014). Similar studies are also known from *Aquilegia* (Whittall and Hodges 2007) or *Mimulus* (Bradshaw and Schemske 2003), which support that shifts in pollinator ecotypes are associated with changes in floral traits. But it is important to state, that while the variations in pollinator environments is an important factor for determining divergent selection, it is by far not the only one (Waser and Campbell 2004; Van der Niet et al. 2014a).

There is growing evidence that non-pollinators (e.g. herbivores, competitors), abiotic factors (e.g. temperature, soil quality) and genetic drift can impose selection and play an important role in shaping floral diversity and speciation (Gavrilets 2003; Waser and Campbell 2004; Strauss and Whittall 2006; Rausher 2008; Gomez et al. 2009; Perez-

Barrales et al. 2013). While pollinator and floral morphologies may strongly overlap, non-pollinator-mediated selection may have acted as original force for the divergence in floral traits (Herrera et al. 2006). More importantly, it has become increasingly clear that floral trait divergence can often be a consequence of non-pollinator-mediated and pollinator-mediated selection working in combination (Waser and Campbell 2004; Strauss and Whittall 2006; Gomez et al. 2009; Schiestl et al. 2011; Kalske et al. 2012; Schiestl 2015). The exact quantification of selection of all actors is therefore essential in distinguishing if floral adaptation is a consequence of pollinator selection or not. Moreover, the quantification of selection has been suggested to often suffer from small sample size as well as lacking reproducibility (Kingsolver et al. 2001). A critical test to determine if floral adaptions are a consequence of local pollinator-mediated selection can be achieved with reciprocal translocation experiments (Agren and Schemske 2012; Boberg et al. 2014; Sun et al. 2014). Such translocation experiments allow the evaluation of plant fitness and the establishment of a relationship of this fitness to pollination success. More specifically, experimental designs where the fitness of certain traits can be separately evaluated can help to identify whether and how pollinators imposed selection (Peter and Johnson 2014). Standardized environments where only pollinators may act as selective agents may also further help to filter and identify strength of pollinator-mediated selection.

Pollinator-driven speciation

One of the central aspects in speciation, at least according to the Biological Species Concept, revolves around the origin and importance of reproductive isolation barriers that prevent gene flow between species in sympatry (Coyne and Orr 2004). Moreover, to understand speciation it is crucial to evaluate the sequence and strength of these individual reproductive barriers (Coyne and Orr 2004; Widmer et al. 2009). Such reproductive barriers are commonly divided up in prezygotic (e.g. temporal, habitat, gametic isolation) and postzygotic (e.g. hybrid inviability, hybrid sterility) barriers (Coyne and Orr 2004). In ecological speciation theory states that divergent selection based on ecological differences will lead to the evolution of reproductive barriers and ultimately speciation (Rundle and Nosil 2005; Nosil 2012). A special case of ecological speciation, in pollinator-driven speciation floral traits diverge under pollinator-mediated selection and adapt towards their local pollinator ecotypes, reproductive

isolation is thought to emerge and reduce gene flow until speciation is complete (Grant and Grant 1965; Stebbins 1970; Coyne and Orr 2004; Waser and Campbell 2004; Johnson 2006). Here pollinators not only act as a selective force for plant traits diversification but can also fulfill an important second role, namely in the reproductive isolation among plants trough floral isolation (Grant 1949; Waser and Campbell 2004; Johnson 2006; Schiestl and Schluter 2009; Xu et al. 2012b; Sun et al. 2015). But while divergence can promote the evolution of reproductive isolation it has also been shown that isolation barriers may emerge trough genetic drift alone (Gavrilets 2003).

Floral isolation

Floral isolation (sometimes described as pollinator isolation), introduced by Grant, consists of two mechanisms that prevent cross-pollination in sympatry, namely isolation trough differences in morphology (mechanical isolation) and by pollinator behavior (ethological isolation), which are often together present (Grant 1949, 1994). Mechanical isolation is based on the flower structure and excellent examples come from *Pedicularis* or *Platanthera* where slight morphological differences cause the different placement of pollen on the pollinators (Grant 1994; Schiestl and Schluter 2009). For ethological isolation, many factors may influence the pollinators' behavior and choice such as color (Bradshaw and Schemske 2003; Hoballah et al. 2007), shape (Whittall and Hodges 2007) and floral scent (Xu et al. 2011; Peakall and Whitehead 2014; Sedeek et al. 2014). Floral isolation often acts in combination with other prezygotic and postzygotic barriers, although the former are often thought to contribute more to total reproductive isolation as they act earlier in time (Coyne and Orr 2004; Rieseberg and Willis 2007; Lowry et al. 2008; Kay and Sargent 2009; Sedeek et al. 2014; Van der Niet et al. 2014a; Sun et al. 2015).

In the context of pollinator-driven speciation it is therefore essential to understand whether the adaptation to new pollinators can promote reproductive isolation trough floral isolation and cause speciation (Van der Niet et al. 2014a). As shifts in pollination systems can differ, it has been suggested, that the likelihood of the evolution of floral isolation may increase under four major aspects: 1) increased pollinator specialization, 2) floral trait divergence in pollinator attractants, 3) shift to different functional pollinator groups, and 4) a sympatric setting (Van der Niet et al. 2014a). The last point is rather counter-intuitive as in general it is assumed that the evolution of floral isolation

occurs under spatial separations while evidence for a sympatric setting is scarce (Coyne and Orr 2004; Waser and Campbell 2004). An exemption may be found in the highly specialized orchids (see next section). The study of isolating barriers of closely related species in sympatry may offer a good approach to quantify the importance of the individual barriers in reproductive isolation (Coyne and Orr 2004). In allopatrically occurring species, translocation experiment may help testing if floral isolation evolved. This will also help us to understand how strong and common floral isolation it is in relation to other pollinator-independent isolation barriers. Nevertheless, pollinator-driven ecological speciation is assumed to have occurred frequently in many independent clades (Van der Niet and Johnson 2012).

Sexual deception: a prime example for pollinator-driven diversification?

In orchids, it has been assumed that the huge floral diversity and species richness is linked to their pollination systems (Cozzolino and Widmer 2005; Tremblay et al. 2005). A feat already proposed by Darwin in his book "*On the various contrivances by which orchids are fertilised by insects*" (Darwin 1877). Moreover, it has been shown that specialization is linked to species richness in orchids, which have an extremely high degree of specialization (Schiestl and Schluter 2009). An excellent study system for the evolution of reproductive isolation and pollinator driven speciation can be found in sexually deceptive orchids (Jersakova et al. 2006; Schiestl and Schluter 2009; Peakall et al. 2010; Xu et al. 2012b; Peakall and Whitehead 2014). In this highly specialized systems plants mimic pollinator females for pollination, and floral isolation has been shown to be often the major (if not only) reproductive barrier between the plants in sympatry (Schiestl 2005; Peakall et al. 2010; Ayasse et al. 2011; Xu et al. 2011; Peakall and Whitehead 2014; Sedeek et al. 2014). Especially the Genus *Ophrys* has been shown to be a good example for pollinator-driven speciation where pollinator shifts occurred often within functional pollinator groups and the traits involved have a simple genetic basis (Schlüter and Schiestl 2008; Xu et al. 2012a; Sedeek et al. 2013; Sedeek et al. 2014; Breitkopf et al. 2015). Moreover, it has been suggested that these processes can occur in sympatry, which is in contrast to the common assumptions that spatial separation is essential (Coyne and Orr 2004; Schlüter et al. 2009; Ayasse et al. 2011). As pollinators are able to learn deceptive plants and to avoid them (Ayasse et al. 2000; Wong and Schiestl 2002), it is assumed that this can reduce the plants fecundity (Tremblay et al.

2005). As a consequence, it has been hypothesized that these plants will be under negative density dependent selection, favoring the switching in pollinators (Schiestl and Schluter 2009; Xu et al. 2011, Chapter 3). All this makes orchids, especially sexually deceptive orchids, prime examples for pollinator-driven diversification even in sympatry.

A new way to study plant adaptation to pollinators?

Although it is commonly acknowledged that pollinators contributed much to floral evolution and plant diversification, details of the processes are still little understood (Johnson 2006). Due to lack of empirical evidence it is still unclear if and how shifts in pollinator environments can create the floral diversity seen today and drive speciation (Johnson 2006; Kay and Sargent 2009; Van der Niet et al. 2014a). This is even more important as climate change, habitat fragmentation and pollinator loss can cause changes in the pollinator environment and ultimately have strong effects on plant-pollinator interactions (Biesmeijer et al. 2006; Schweiger et al. 2010). Thus the study of plant adaptations to pollinators and the processes involved are crucial.

There are several classical approaches to study plant adaptations to pollinators. Indirect evidence for pollinator-driven adaptation is often provided by studies showing correlations between floral traits and pollinator environments (Anderson and Johnson 2009; Newman et al. 2014; Van der Niet et al. 2014b; Gomez et al. 2016). Measurements of pollinator-mediated selection and linking it with the plant phenotype represent another approach (Galen et al. 1987; Medel et al. 2003). A powerful tool for identifying adaptations to pollinators is the usage of translocation experiments (Boberg et al. 2014; Runquist and Moeller 2014; Sun et al. 2014). Plants adapted to their local pollinators should suffer a reduced fitness in a different setting where pollinator environments are different.

We have taken a novel approach to understand the process of plant adaptations to pollinators by using an experimental evolution approach. Experimental evolution, defined as "the study of evolutionary changes occurring in experimental populations as a consequence of conditions (environmental, demographic, genetic, social, and so forth) imposed by the experimenter" (Kawecki et al. 2012). Such studies are well known from bacteria where short generation time and controlled environments allow the observation of selection, adaptation and speciation in real time (Lenski et al. 1991).

However, experimental evolution studies on plant-pollinator interactions are extremely scarce and commonly not applied to this topic. Such an approach with constant environmental conditions but changing pollinator environments can help us to measure the effects that pollinator shifts can have on selection, plant trait evolution and reproductive isolation in real-time. Constant environmental conditions with the exception of the pollinator environments would give us deep insights in the processes following a shift in pollinators. Moreover, controlled changes in abiotic factors (e.g. light, temperature, nutrient) as well as non-pollinators (e.g. herbivory) will help us additionally to understand how strong these individual factors influence floral evolution and plant speciation (Angert et al. 2008; Agrawal et al. 2012).

A rare example can be seen in a study on *Mimulus gutattus* where in an experimental evolution approach the effects of pollinator loss were analyzed over multiple generations (Roels and Kelly 2011). A plant family, which offers a good system for evolutionary studies, can be found in the *Brassicacea* (Gomez et al. 2016). Specifically, promising plant candidates for applying experimental evolution in a plant-pollinator context can be found in the rapid-cycling populations of *Brassica.* These plants have an extreme short generation time, generalized pollination system, can be easily grown in the greenhouse and represent economically important crops (Williams and Hill 1986; Rader et al. 2009; Rader et al. 2013). Moreover, the taxonomic closeness to *Arabidopsis thaliana* (Al-Shehbaz et al. 2006), the model system for identification of genes and their function, makes these plants a convincing case for experimental evolution studies on plant-pollinator interactions.

Aim and chapters in this thesis

The aim of this thesis is the study of pollinator-mediated mechanisms that drive floral trait evolution and plant speciation. For this I studied the effects of changing pollinator environments and different efficient pollinators on plant trait evolution in an experimental evolution approach, using a fast growing *Brassica* with a generalized pollination system. Additionally, to study pollinator adaptation and the importance and strength of floral isolation I quantified the individual barriers in two closely related sexually deceptive orchids. The thesis is structured into three Chapters.

In Chapter I, I focus on the question: What are the effects in pollinator shifts on plant trait evolution? For this I use an experimental evolution approach with rapid-

cycling *Brassica rapa* plants, having a generation time of two months, and bumblebees (*Bombus terrestris*) and hoverflies (*Episyrphus balteatus*) as pollinators. I created 108 full sib seed families by artificial crossing and these were used as the starting population for the experiments. Two populations were created of which a subset was allowed to be visited bumblebees and hoverflies respectively. These visited plants were able to set seeds which then were used to grow the next generation. Every second generation, I measured a wide range of floral traits, i.e. floral scent, flower color and flower morphology. Additionally, reproductive success, pollinator efficiency, number of pollinator visits and pollinator choices were measured to quantify pollinator-mediated selection and floral trait evolution over time in each pollinator treatment. After 9 generations I also quantified if reproductive isolation emerged between the plants of the different pollinator treatments.

In Chapter II, I addressed Stebbins' principle of the most effective pollinator and ask the question: Does the most effective pollinators impose the strongest selection on floral traits? I used the similar experimental setup as in Chapter I but with the differences that both pollinators were simultaneously present and allowed to visit the plants (sympatric setup). I quantify if the more efficient pollinator and its preferences dominate pollinator mediated selection and floral trait evolution.

In Chapter III, the key question is: Which are the key reproductive barriers in the two closely related European sexually deceptive orchids *Ophrys insectifera* and *O. aymoninii*? For this I measured pollinator-mediated floral isolation trough quantification of pollen flow between the two species and analyzed the floral scent of both species. Further, in behavioral essays the importance of key scent compounds in floral isolation were quantified. Trough intra-and interspecific crosses I quantified postpollination and postzygotic barriers. In addition, the mycorrhizal associations of both species were analyzed for specialization to specific fungi.

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Source Fig 1: Frontispiece of C.K. Sprengels book "*Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen"* from <http://dx.doi.org/10.3931/e-rara-14571>[State: 26.10.2016].

Chapter I

Effects of changing pollinator environments on plant trait evolution; an experimental evolution approach

Daniel D.L. Gervasi, Florian P. Schiestl

Left: *Brassica rapa* plant after nine generations exposed to a bumblebee environment, and left: *B. rapa* plant after nine generation exposed to a hoverfly environment.

Abstract

Selection mediated through pollinators played a key role in the floral adaptation of plants. Although the importance of pollinator-driven selection on plant evolution cannot be neglected, it has never been demonstrated in an experimental approach where the pollinator environment has been manipulated. In our study we experimentally changed the pollinator environment in *Brassica rapa* (Wisconsin rapid cycling) plants to investigate pollinator-mediated selection and the evolutionary consequences on plant traits and fecundity over several generations. As pollinators we used the bumblebee, *Bombus terrestris*, and the hoverfly, *Episyrphus balteatus*, to which the plants were exposed separately. We found that bumblebees mediated significant positive selection on floral scent as well as morphological traits, while hoverflies mediated weak selection. In terms of evolutionary response, we found that over 60 % of the plant traits showed evolutionary changes after nine generations. Notably scent emission was found to increase strongly in bumblebee plants, while plant height decreased strongly in hoverfly plants. Additionally the pollinator efficiency and fecundity of the plants differed significantly; bumblebees were much more efficient pollinators than hoverflies and plants pollinated by bumblebees also had a higher fecundity. However, after nine generations only in the hoverfly treatment the pollination efficiency and fecundity in the plants increased, indicating adaptations to the low efficiency of these pollinators. This study thus shows for the first time the effects of changing pollinators on plant evolution in an experimental approach over several generations.

Introduction

Pollinators play an essential role in the reproduction of angiosperms as approximately 87 % of all flowering plants are animal-pollinated (Ollerton et al. 2011). In addition, pollinators provide essential economically services to humans through pollination of crop plants (Klein et al. 2007; Gallai et al. 2009). However animal pollinators provide not only pollination services for plants but also impose selection on a multitude of floral traits based on their preferences (Kingsolver et al. 2001; Fenster et al. 2004; Waser and Campbell 2004; Hoballah et al. 2007; Schiestl and Johnson 2013). Overall, it has been shown that floral traits represent adaptations toward pollinators (Harder and Johnson

2009), and have been suggested to alleviate pollen limitation (Johnson 2006). It is therefore commonly acknowledged that pollinator-mediated selection is one of the main forces in floral adaptation. The importance of understanding the process of pollinator-mediated selection and floral adaptations has become even more apparent in the last decades as the impacts of climate change, habitat fragmentation and invasions of non-native species on plant-pollinator interactions increased (Kearns et al. 1998; Memmott et al. 2007; Hegland et al. 2009). These factors often encompass the decline or loss of pollinators, which can affect plant reproductive success and mating systems (Robertson et al. 1999; Memmott et al. 2004; Biesmeijer et al. 2006; Eckert et al. 2010; Brosi and Briggs 2013). Furthermore, the increase of temperatures caused by climate change (IPCC 2007) can lead to shifts in the pollinator assemblages as pollinators often have fairly specific climatic requirements (Parmesan et al. 1999; Wilson et al. 2007; Schweiger et al. 2010; Kerr et al. 2015). Such shifts may have profound effects on plant-pollinator interactions (Biesmeijer et al. 2006; Schweiger et al. 2010), but the consequences on pollinator-mediated selection and the corresponding adaptive evolution of reproductive plant traits mostly remain theoretical, as experimental data are scarce.

An important research approach for studying evolutionary processes is experimental evolution (Kawecki et al. 2012). This approach enables the real-time study of evolutionary changes in biological systems under controlled environmental conditions. Although experimental evolution in plant-pollinator interactions has up to now rarely been studied, evolutionary effects of experimental pollinator loss on plant evolution have recently been studied in *Mimulus guttatus.* In this study, it was shown that pollinator loss can cause a rapid evolutionary change in the plant mating system towards increased self-fertilization (Roels and Kelly 2011), thus supporting the theory that plants evolve increased self-fertilization under pollinator limitation (Lloyd 1992). In contrast, the effects of pollinator shifts on floral evolution have, to our knowledge, never experimentally been examined. Different pollinator assemblages are thought differ in the selective pressure they impose on plants, as well in the efficiency of pollination, as different pollinators have different morphologies and preferences (Fenster et al. 2004; Waser and Campbell 2004). These differences in pollinator environments coupled with the differences in the selection regime, have been used as a basic model for explaining floral diversification and plant speciation (Johnson 2006, 2010). Therefore, shifts in pollinator assemblages can change the selection regime on

plants and may have strong impacts on plant trait evolution and speciation. However, due to lack of empirical evidence it is still unclear if and how shifts in pollinator environments can create the floral diversity seen today and drive speciation (Van der Niet et al. 2014).

In our study we use for the first time an experimental evolution approach to examine the effects of pollinator change on plant trait evolution and incipient reproductive isolation. We use *Brassica rapa* plants (Wisconsin Fast Plants®), which are outcrossing with a generalized pollination system and have a short generation time (1.5 month). Trough exposure to different pollinators (bumblebees and hoverflies) and random hand pollination (as control) under standardized environmental conditions in the greenhouse we imitated a change in the pollinator environment. Over the course of nine generations we quantified pollinator-mediated selection and plant trait evolution. We used the data to answer the following questions: (i) Do pollinators differ in the pollinator-mediated selection they impose on plants? (ii) Do plant traits and mating system evolve in response to the selective pressures imposed by the pollinators? (iii) Do the plants adapt to the different pollinators through increased fecundity? (iv) Do we see the emergence of reproductive isolation due to adaptations to different pollinators?

Material and Methods

Study Systems

In this study the model plant *Brassica rapa* (Wisconsin Fast Plants®) was used. These outcrossing plants have a extremely short generation time of about 35-40 days (Tomkins and Williams 1990). 300 seeds (Wisconsin Fast Plants® Standard Seed) were obtained from Carolina Biological Supplies (Burlington, NC, USA), and grown in a phytotron under standardized soil, light and watering conditions. From these 300 plants 108 full sib seed families were generated by artificial crossing (only seed families from crosses were both parents produced fruits were used). These 108 full sib seed families were used as the starting population for the experiment. In the two pollinator treatments, plants were either exposed to bumblebees (*Bombus terrestris,* Biocontrol, Andermatt, Switzerland) or hoverflies (*Episyrphus balteatus,* Katz Biotech AG, Germany). Both insect species are well known pollinators of Brassicacea plants (Jauker and Wolters 2008; Rader et al. 2009) and differ strongly in their morphology but also may vary in

their sensory system (Briscoe and Chittka 2001). In the control treatment, randomly chosen plants were hand pollinated.

In the starting population (generation 1), a member of every seed family was used in each of the three pollinator treatments to control for genotype among treatments. Each treatment therefore consisted of 108 plants (representing 108 seed families), which we subdivided into three replicates (A, B, and C) each containing 36 plants of one family each (Fig S1). The plants of all replicates in all the treatments were grown in the phytotron under standardized soil (Einheitserde® classic, Einheitserde Werkverband e.V., Germany), light (24hrs light) and watering conditions. All plants were phenotyped every second generation starting with generation one.

Plant traits

All morphological traits were measured before pollination. Petal width, length, stigma height and flower diameter of three randomly chosen flowers per plant were measured with an electrical caliper (Digital Caliper 0-150 mm,TOOLCRAFT®). Nectar from three flowers was collected with 1 μl glass capillaries (Blaubrand, Wertheim, Germany) and the volume determined by measuring the length of nectar in the micropipette with an electrical caliper. For the analysis the mean of each of the traits from the three flowers was used. Additionally, the number of open flowers and height of each plant were recorded before pollination (but on the same day).

Floral volatiles were collected before bioassays in a nondestructive way from all plant inflorescences as soon as at least five flowers were open. We used headspace sorption with a push-pull system identical to Schiestl et al. (2014). The inflorescences of the plants were enclosed in glass cylinders previously coated with sigmacote (Sigma-Aldrich) and closed with a Teflon plate. The number of open flowers was noted for each plant. Air from the surrounding was pushed with a flow rate of 100 ml min^{-1} trough activated charcoal filters into the glass cylinder. Simultaneously, air was pulled from the glass cylinder with a flow rate of 150 ml min^{-1} trough a glass tube filled with ~30 mg Tenax TA (60/80 mesh; Supleco, Bellefonte, PA, USA). Air from empty glass cylinders was collected as air control. Floral volatiles were collected for two hours in the phytotron under standardized light and temperature conditions. Scent collection was obtained from generation 4, 5, 7 and 9. Floral scent data from generation 1 and 3 was lost due to technical problems; instead, scent was collected from generation 4. Floral

scent data of generation 1 was obtained after the end of the experiment by re-growing plants from the starting generation and collecting scent from one plant from each of the 108 seed families. Thus, from the first generation in total 108 plants (36 from each replicate) were sampled for floral scent. Chemical analysis and quantification were based on the methods described in Schiestl et al. (2014). In detail, for the analysis of floral scent, gas chromatography with mass selective detection (GC-MSD) was used. Samples were injected into a GC (Agilent 6890 N; Agilent Technologies, Palo Alto, CA, USA) by a MultiPurpose Sampler (MPS; Gerstel, Müllheim, Germany) using a Gerstel thermal desorption unit (TDU; Gerstel) with a cold injection system (CIS; Gerstel). For thermodesorption, the TDU was heated from 30 to 240°C at a rate of 60°C min^{-1} and held at a final temperature for 1 min. The CIS was set to -150°C during the trapping of eluting compounds from the TDU. For injection, the CIS was heated to 250°C at a rate of 12°C s^{-1}, and the final temperature was held for 3 min. The GC was equipped with a HP-5 column (0.25 mm diameter, 0.25 um film thickness, 15 m length), and helium was used as carrier gas at a flow rate of 2 ml min^{-1}. Compound identification and quantification were done following Schiestl et al. (2014) with the Agilent MSD ChemStation Program. Quantification of compounds was obtained through measurement of peak areas of selected target ions specific to the individual scent compounds. Specific target ions were obtained from synthetic standards of all compounds. Only scent compounds that were present in significantly higher amounts than in the air control were included in the analysis (in total 14 scent compounds). All amounts of VOCs were calculated in ρg per flower l^{-1} sampled air.

After pollination (but on the same day) the color reflectance spectra of three petals from different flowers per plant were recorded using a fiberoptic spectrophotometer (AvaSpec-2048; Avantes, Apeldoorn, the Netherlands) and a Xenon pulsed light source (AvaLight-XE; Avantes). One petal at a time was placed under the spectrophotometer (upper part of the petal which is closest to the corolla) and the percentage reflectance between 200nm and 900nm every 0.6nm was recorded in transmission mode. Of the spectrum measured, only the mean of the reflectance values every 10nm from 260nm to 650nm from the three petals were used in the analysis. Across the entire experiment, plant traits of a total 1983 plants were collected and analysed (Bumblebee: $N_{morphology\ and\ nectar} = 524$, $N_{scent} = 414$, $N_{color} = 525$; Hoverfly: $N_{morphology\ and\ nectar} = 509$, $N_{scent} = 384$, $N_{color} = 503$; Control: $N_{morphology\ and\ nectar} = 535$, $N_{scent} = 426$, $N_{color} = 535$; Generation 1: $N_{scent} = 107$).

In the first and ninth generation, nectar sugars were analyzed; the nectar of ~3 flowers of one plant were taken from at least ten different seed families of every replicate and treatment in the ninth generation (Bumblebee N = 39, Hoverfly N = 42, Control N = 40). From first generation plants re-grown for scent collection, nectar sugar was additionally collected from at least ten different plants (each representing a different seed family) of every replicate (N = 36). In total nectar sugars of 157 plants were collected. Nectar sugar analysis was conducted as described in Knauer and Schiestl (2015). The nectar was collected by 1µl micropipettes (Blaubrand, Wertheim, Germany) on blotting paper and the sugar was later dissolved into 1ml Milli Q water for 90 min at 60°C and 400 rpm. 200ul of the sugar solution was transferred into a 2ml glass vial and dried over night at 60°C in the oven. After drying 100ul TDS solution, which was previously prepared by mixing anhydrous pyridine (Fisher Chemical), hexamethylsilazane and trimethylchlorosilane (Sigma Aldrich) under oxygen and water exclusion at a 10:5:3 ratio, was added for derivatisation and 30 µl were transferred into vials for GC-MS. For quantitative analysis, gas chromatography with mass selective detection (GC-MSD) was used. Of every sample 1µl was injected into a GC (Agilent 6890 N; Agilent Technologies, Santa Clara, CA, USA) by a MultiPurpose Sampler (MPS; Gerstel, Müllheim, Germany) at 65° (2min), followed by a programmed increase of the oven temperature to 300°C at a rate of 6°C min^{-1} and held at the final temperature for 7 min. The GC was equipped with a HP-5 column (0.25 mm diameter, 0.25 µm film thickness, 15 m length), and helium was used as carrier gas at a flow rate of 2.0 ml min^{-1}. Compounds were identified by comparison with retention times and spectra of synthetic standard compounds. Quantification was performed as described in the VOC analysis section. For statistical analysis the total sugar amounts per flower were calculated as the sum of all different sugars (fructose, glucose and sucrose).

To assess the changes in total leave glucosinolates amounts in the plants, leave samples (~100 mg fresh weight) of one member of every seed family in the first and ninth generation were taken and immediately frozen in liquid nitrogen. Samples were weighed (ca. 100 mg) and ground to a fine powder with a Retsch Mixer mill using 3 metal balls. The samples were grinded for 1 minute and cooled down again in liquid nitrogen before grinding them another minute at high speed. To the fresh grinded powder we added 1ml of a solution of sinalbin (5µg ml^{-1}; internal standard) and ice cold MeOH:water (70:30 ; Methanol gradient grade 235nm). Samples were vortexted for 5 s and immediately incubated at 85°C for 10 min in a block heater and simultaneously

mixed with 600 rpm (Eppendorf Thermomixer® comfort). For further extraction, samples were put in an ultrasonic bath for 10 min (Advantage-Lab, Typ AL 04-04). Extracts were then centrifuged at 14000 rcf for 10 min (*Sorvall RMC 14, Kendro Laboratory* Products (USA)) and the supernatant was transferred to a new tube and stored at -20°C until UHPLC analysis. UHPLC/MS analysis and quantifications were performed identical as in Schiestl et al. (2014).

Pollination treatments

Exposure to pollinators was performed in a flight cage (2.5m x 1.8m x 1.2m) in the greenhouse under standardized light conditions with bumblebees and hoverflies separately. Experiments were performed between 9am and 3pm from January 2013 till November 2014, using plants 23 days post sowing out. Bumblebees were purchased from Biocontrol (Andermatt, Switzerland) as complete hives and held in a separate flight cage in the greenhouse. Hoverflies were purchased as pupae (Katz Biotech AG, Germany) and reared until eclosure after which male and female flies were separated. Pollinators were allowed to visit and feed on flowering *B. rapa* plants (Wisconsin Fast Plants®). Additionally they were fed with supplemental pollen (Biorex, Ebnat-Kappel, Switzerland) and supplemental sugar water until three days prior to behavioral experiment after which only supplemental pollen and sugar water was used. Supplemental sugar water and pollen were removed 16 hours before pollination.

For pollination, plants in each replicate were randomly placed in a square of 6 x 6 plants with a distance of 20 cm from each other in the flight cage. Pollinators were added individually and each insect was allowed to visit a maximum of three different plants and removed from the cage afterwards; each insect was only used once. For hoverflies both male and females were, if possible, used in equal quantities and for bumblebees only workers were used. In total, 12-15 plants per replicate received (a) visit(s) by pollinators. For the plants that were visited, the number of visits and number of visited flowers was recorded. In the control group, 12 plants were chosen randomly per replicate and five flowers of each plant were hand pollinated by randomly chosen father plants of the same 12 plants. Each plant could be pollen donor to more than one plant but only receive pollen from one plant. After pollination, visited flowers were marked and plants were kept in a cage for additional 30 days until the fruits were harvested. Seed set (fecundity) of each visited plant was measured and relative seed set

was calculated for each plant by dividing the individual seed set by the mean seed set in the replicate. The average seed weight for each visited plant was also mesured. Additionally, pollination efficiency as number of seeds per fruit was calculated for each visited plant. From all seeds produced by the pollinated flowers, a number of seeds representative of the individual seed production were used to grow the next generation. The germination rate oft the seeds was additionally quantified for each replicate in every treatment and generation (for the last generation the germination rate was not quantified). The seed contribution of an individual plant into the next generation was correlated to the fecundity. The more seeds a plant produced the more seeds it contributed to the next generation, which consisted of 36 plants for each replicate. The seed contribution of each visited plant into the next generation was calculated for every replicate as: (36* individual seed set)/replicate sum of seeds. Values below 0.5 were rounded up to 1.

Reproductive isolation

In the last generation reproductive isolation was measured between bumblebee- and hoverfly-selected plants through behavioral assays with pollinators and plant crosses. Seeds from all seed families of the ninth generation were used to grow the plants for pollinator preference assays and inter/intra-treatment crosses. For floral isolation and crossing experiments between replicates within a treatment, seeds from the tenth generation were used. This was due to a shortage of seeds of ninth generation plants. The plants were grown in the phytotron under standardized light and water conditions for 23 days. Assays and crosses were always conducted with bumblebee and hoverfly plants from the same replicate (e.g. replicate A bumblebee plants were crossed with replicate A hoverfly plants).

Pollinator preference

Assays for pollinator preferences were conducted for each replicate with both types of pollinators. For each replicate two behavioral assays were performed (one for each pollinator treatment). Each behavioral assay consisted of 36 plants from the ninth generation; half of them bumblebee plants and the other half hoverfly plants. The plants were positioned by alternating bumblebee and hoverfly plants, in a square of 6 x 6

plants with a distance of 20 cm from each other in the flight cage (2.5m x 1.8m x 1.2m). For every essay only one pollinator was allowed to enter and visit the plants at a time. In total 30 bumblebees and 56 hoverflies were used in all assays. First choice of each pollinator was recorded and used for calculation of the pollinator preference.

Floral isolation (FI)

For floral isolation, plants of five seed families per replicate from the hoverfly and bumblebee treatment of the tenth generation were grown in the phytotron (in total 150 plants). After 23 days, dual-choice assays were performed in the greenhouse. A bumblebee plant and hoverfly plant were placed at a distance of 40 cm from each other in a flight cage (2.5m x 1.8m x 1.2m). Only bumblebees were used in these assays as they showed clear preferences for bumblebee plants in contrary to hoverflies, which showed no preference. A bumblebee was then released into the cage and allowed to visit one plant. After landing the non-visited plant was removed and replaced with a new pair of bumblebee- and hoverfly plant at a distance of 40 cm. The bumblebee was then allowed to visit one of the two newly added plants. After the bumblebee landed on a plant, it was removed and stored separately to prohibit repeated usage. In total 34 bees were assayed for all replicates. Based on the number of switches within and between bumblebee and hoverfly plants (intra- and inter"specific" visits), the floral isolation index was calculated after Sobel and Chen (2014) for each replicate as: FI=1-(#interspecific visits/#intraspecific visits). A value of 0 or negative means no floral isolation and 1 means complete floral isolation. For analysis the mean floral isolation index of all replicates was used.

Post-pollination reproductive isolation (PPI)

For post-pollination isolation inter- and intra-specific crosses were performed for every replicate. Interspecific crosses were obtained by crossing (in both directions) 3-5 flowers of bumblebee- and hoverfly plants representing all seed families from the ninth generation (in total 60 plants). For intraspecific crosses the same number of flowers were crossed within each treatment group and replicate (in total 68 plants). Fruit development, number of seeds and seed weight were measured for each plant. Post-pollination isolation was then calculated after Scopece et al. (2007) for each replicate in

the bumblebee and hoverfly plants as: 1-(average seeds per interspecific cross /average seeds per intraspecific cross). A value of 0 or negative means no isolation and 1 means complete isolation. Total reproductive isolation was calculated after (Ramsey et al. 2003) as $R_{total}= R_{Floral} +(R_{PPI}(1-R_{Floral}))$. For the analysis the mean value of the replicates was used. Additionally, to discriminate treatment-specific isolation from random, replicate-specific isolation, we performed crosses between the replicates within treatments. This was performed in three flowers in bumblebee and hoverfly plants from 3-5 seed families per replicate and on average 10 individuals per replicate (in total 63 plants). Post-pollination isolation between replicates was calculated as described above and the mean value was used for analysis.

Self-compatibility and autonomous-selfing

To test for self-compatibility in the first and ninth generation, we selfed on average three flowers from a total of 103 plants grown from each seed family of generation 9, in all treatments and all replicates (Bumblebee: N = 34, Hoverfly: N = 35, Control: N = 34. For generation 1, randomly chosen plants from different seed families in every replicate (in total 40 plants) were re-grown after the experiment and then selfed. Mean number of seeds per selfed flower for each individual plant was used as measurement for self-compatibility.

To test for autonomous-selfing, we allowed eight plants (each plant representing a seed family) from every treatment and replicate of generation 8 to grow without disturbance (Bumblebee: N = 22, Hoverfly: N = 24, Control: N = 23). Generation 8 was used due to a shortage of seeds of the ninth and tenth generation. Simultaneously, we randomly selected 8 plants (each plant representing a seed family) from every replicate of generation 1 and re-grew them without disturbance (in total 24 plants). After 23 days the remaining buds in each plant were cut and number of opened flowers was recorded. The plant was then allowed to ripen their fruits, which were also recorded. The number of seeds per open flower was used as a measurement for autonomous-selfing for each plant.

Statistics

Phenotypic selection:

Selection differential (S) and gradients (β) were calculated similar to (Huber et al. 2005; Parachnowitsch et al. 2012) with univariate (selection differential) and multivariate (selection gradient) generalized linear models (glm) as the covariance between plant trait(s) and plant fitness (for clarification see below). For the multivariate models, the plant trait variables were divided into three groups: a) morphology and nectar (flower number, plant height, stigma height, petal length and width, flower diameter, nectar per flower), b) color (wavelengths 260nm-650nm) and c) floral scent (14 scent compounds) which were individually analyzed as they were each not always available for every measured generation. For the analyses of scent amounts and nectar volume, data were ln(1+x) transformed to approach normal distribution of the data. All variables were then z-transformed to a mean of 0 and standard deviation 1 on the replicate level in each treatment and generation. To reduce the high number of color variables, additionally a principal component analysis (PCA) with varimax rotation was performed on the standardized color variables. Only principal components (PCs) with an Eigenvalue above one were used in the subsequent analysis. For floral color the PCA resulted in four PCs explaining 97.261% of the total variance. For the measurement of selection, the data of all replicates and generations were combined in each treatment to increase statistical power. Selection differential were calculated by univariate glm (poisson distribution with log link) for each trait in every treatment (differentials include direct selection on a trait and selection via correlated traits). Selection gradients, which measure only direct selection on a trait (Lande and Arnold 1983), were then calculated for every treatment and trait-group separately by multivariate glm (poisson distribution with log link) with fitness as dependent variable and traits as covariates. For measuring pollinator-mediated selection over all generations "number of visits" was used as fitness variable, which strongly correlated with rel. seed set (r_{1233}=0.648, p < 0.001). Rel. seed set was not used as fitness in this analysis, as the values were strongly zero inflated, which was not true for "number of visits". To check for differences in pollinator-mediated selection gradients between bumblebees and hoverflies, a multivariate glm (poisson distribution with log link) with fitness (number of visits) as dependent variable, treatment as fixed factor, plant traits as covariates and the interaction treatment x plant trait was performed for each trait-group.

Evolutionary change:

Evolution of traits over generations was measured as linear regression with the trait as dependent variable and "generation" as independent (explanatory) variable. This was done for each treatment and replicate separately. Evolutionary changes of a trait over time were only adopted if the changes were significant in a regression analysis in either all three replicates (consistent evolution) or two replicates (less consistent evolution), and followed the same direction (regression coefficient either always positive or always negative). An additionally criterion was that traits needed to have undergone divergent adaptation. This was based on a general linear model in the ninth generation with the condition that the factor "treatment" or the interaction "treatment*replicate" (or both) were significant different between the treatments. Trait differences between the treatments were analyzed by univariante general linear models (full factorial) and Bonferroni *post-hoc* tests with the trait as dependent variable, treatment as fixed factor and replicate as random factor in the first and ninth generation separately. For the analyses of scent amounts and nectar volume, data were ln(1+x) transformed to approach normal distribution of the data. For the general linear model with the color variables, a PCA was performed as described above but without first standardizing the variables. The PCA was performed for all treatments, replicates and all generations together resulting in four PCs explaining 96.9 % of the total variance. For fecundity and pollination efficiency only values of visited plants were used. An exception was the trait seeds per visited plants (fecundity), where linear regression and general linear models were only performed with the bumblebee and hoverfly treatment. Evolutionary changes in plant traits that fulfilled all above-mentioned criteria and also differed between the pollinator groups were considered to be generated by the different pollinator environments. However, if the evolutionary changes were identical in all pollinator treatments, these changes were not attributed to different pollinator environments. Additionally, evolutionary changes in traits that did not fulfill all the above-mentioned criteria were considered to be generated by drift.

Discriminant function analysis was performed with floral scent data from the first and the ninth generation (N = 422). Discriminant analysis of flower morphology (petal length and width, flower diameter, stigma height) between the treatments was also performed in the first (N = 322) and ninth (N = 319) generation separately. To test for different amounts of glucosinolates per plant between the treatments a oneway ANOVA was used. For differences within a treatment between generation 1 and 9 a student's t-

test was used. Sugar amount per flower were analyzed by oneway ANOVA over generation 1 and 9 (replicates combined).

Seed quality:

The average seed weight per visited plant was compared among the three treatments over all replicates and generations by a one-way ANOVA with LSD *post-hoc* test. The seed germination rate was also compared among the three treatments over all generations and replicates combined by a one-way ANOVA with LSD *post-hoc* test.

Mating system and reproductive isolation:

Self-compatibility (mean number of seeds per selfed flower) was analyzed between generation 1 and each treatment in generation 9 by a general linear model (full factorial) with LSD *post-hoc* test. Self-compatibility (square root +1 transformed to approach normal distribution) was used as dependent variable, treatment as fixed factor and replicate as random factor. Autonomous-selfing (mean number of seeds per open flower) in generation 8 between the treatments and generation 1 was analyzed by general linear model (full factorial) with LSD *post-hoc* test. Autonomous-selfing (ln+1 transformed to approach normal distribution) was used as dependent variable, treatment as fixed factor and replicate as random factor. The first choice preferences of bumblebees and hoverflies were analyzed by binomial test (test-prop = 0.5) for which all replicates have been pooled to increase sample size. To test if floral isolation for bumblebee pollinators was significantly different from zero (no floral isolation) a one-sample t-test was performed. Post-pollination isolation between hoverflies and bumblebees was analyzed with one-sample t-tests to test if it was significantly different from zero (equal reproductive success) over all replicates. To test if post-pollination isolation between treatments is higher than between replicates within a treatment we used simple t-test.

Statistics were performed with IMB SPSS Statistics (Version 22.0.0, http://www-01.ibm.com/software/analytics/spss/products/statistics/).

Results

Pollinator-mediated selection

Bumblebees were found to impose more frequently significant pollinator-mediated selection on plant traits compared to hoverflies or random hand pollination (Table S1). Overall, pollinator-mediated selection was found to be present in plant morphology and floral scent but not in flower color.

Significant selection differentials were found in all treatments (Table S1). Within plant morphology, in all treatments flower number and petal length were under significant positive selection (Table S1). Bumblebees and hoverflies also showed significant selection for plant height and flower size (Table S1). Additionally bumblebees selected also significantly for increased petal width (Table S1). Floral scent compounds were found to be mostly under selection in the bumblebee treatment and less in the hoverfly or in the control group (Table S1). Bumblebees selected plants with increased emission in aromatics and nitrogen containing aromatics, specifically methyl benzoate, p-anisaldehyde, indole and methyl anthranilate (Table S1). The sulphur containing compound 1-butene-4-isothiocyanate however was found to be under significant negative selection by bumblebees. Hoverflies only showed significant selection for lower emission of (*Z*)-3-hexenyl acetate and in the control group significant selection for increased α-farnesene was detected (Table S1).

The multivariate analysis showed similar but not identical patterns in the selection gradients. Bumblebees were found to exert significant positive directional selection on plant height, petal length and flower number while hoverflies showed only significant positive selection gradients on flower number (Fig 1). No selection on morphological traits was found in the control treatment (Table S1). Selection of floral scent was found to be almost exclusively in the bumblebee treatment. Within the aromatics bumblebees imposed significant positive selection on methyl benzoate and *p*-anisaldehyde, while negative selection was found on benzaldehyde (Fig 1, Table S1). Additionally, in the nitrogen-containing aromatic compounds bumblebees imposed significant positive selection on indole and strong negative selection on benzyl nitrile (Fig 1, Table S1). However, an exception was a-farnesene which was under significant positive selection in the control treatment (Table S1). Hoverflies showed no selection on any scent compounds (Table S1). Comparisons of the selection gradients on morphology between bumblebees and hoverflies showed that selection gradients were significant different in petal width and plant height (Fig 1, Table S2). Within floral scent, selection gradients

were found to differ significantly in methyl benzoate, benzyl nitrile and *p*-anisaldehyde (Fig 1, Table S2).

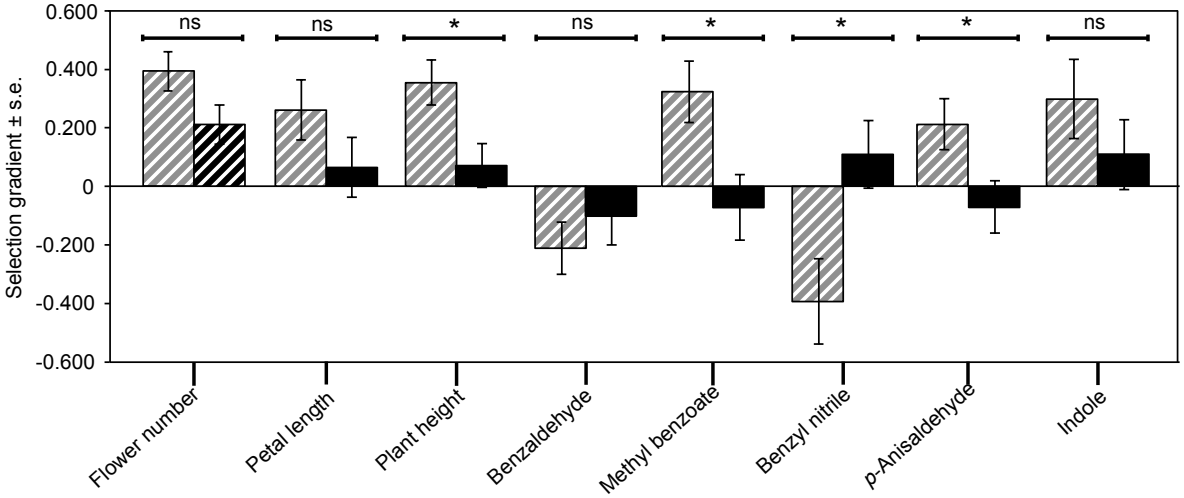

Fig 1: Phenotypic selection gradient (± s.e.) on plant traits in bumblebee (grey) and hoverfly treatment (black). Only traits in which significant selection gradients by either pollinator treatment is found (marked bars) are shown (glm, $p < 0.05$). Asterisks above the bar indicate significant difference in selection between the pollinator treatments (glm, $p < 0.05$).

Morphology, color and scent evolution

During the 9 generations of our evolution experiment, a wide variety of plant traits have undergone evolutionary changes in the different pollinator treatments. A summary of all traits that have undergone evolutionary changes can be found in Table 1. As expected, plant traits in the different treatments did not differ from each other in the first generation, except in color (Fig 2A, function 1-2, Wilk's Lambda=0.984, χ_8^2=4.986, p=0.759;function 2, Wilk's Lambda=0.995, χ_3^2=1.712, p = 0.643; Table S3).

Table 1: List of all plant traits that fullfilled the criteria for evolutionary changes and divergent adaptations in the three treatments. Significant positive/negative linear regression in all three replicates are shown as +++/--- and in two replicates are shown as +/-. No evolutionary changes are shown as 0. Significant differences between treatments based on general linear model (GLM) in the 9th generation are show as "t" when factor "treatment" was significant and ♯ if interaction "treatment x replicate" was significant.

Trait	Treatment	Regression	GLM	Trait	Treatment	Regression	GLM
Morphology and nectar				Nitrogen containing aromatic			
Flower number	BB	+		Benzyl nitrile	BB	+	
	HF	0	♯		HF	0	♯
	CO	0			CO	0	
Petal length	BB	0		2-Aminobenzaldehyde	BB	0	
	HF	0	t,♯		HF	-	♯
	CO	+++			CO	-	
Petal width	BB	0		Indole	BB	+	
	HF	-	♯		HF	0	♯
	CO	0			CO	0	
Flower diameter	BB	0		Methyl anthranilate	BB	+++	
	HF	0	t		HF	0	t
	CO	+			CO	0	
Plant heigth	BB	0		Total amount of scent per flower			
	HF	---	t,♯	Sum Scent	BB	+++	
	CO	0			HF	+	♯
Nectar per flower	BB	0			CO	0	
	HF	+	♯	**Color**			
	CO	0		PC1 (290-400nm)	BB	0	
Floral Scent					HF	---	♯
Aromatics					CO	---	
Benzaldehyde	BB	+++		PC3 (410-490nm)	BB	+	
	HF	+++	♯		HF	0	♯
	CO	+++			CO	+	
Phenylacetaldehyde	BB	0		PC4 (260-280nm)	BB	+++	
	HF	-	♯		HF	+++	♯
	CO	0			CO	+++	
Methyl benzoate	BB	+++		**Seed production**			
	HF	+	♯	Seeds per fruit	BB	0	
	CO	+			HF	+	t,♯
p-Anisaldehyde	BB	+			CO	0	
	HF	0	t,♯	Seeds per visited plant	BB	0	♯
	CO	0			HF	+++	
Fatty-acid derivates							
(*Z*)-3-Hexenyl acetate	BB	+++					
	HF	+	♯				
	CO	0					

With respect to morphological traits and nectar production, we found evolutionary changes to occur in the treatments (Table 1). Flower number was found to increase in the bumblebee treatment while remained constant in the other treatments (Table S4). Plant height and petal width decreased in the hoverfly treatment but not in the other two treatments (Fig 2B; Table S4). Petal length and flower diameter were found to increase in the control treatment while in the two other treatments these traits remained unchanged (Table S4). Additionally, the amount of nectar produced per flower increased in the hoverfly plants but not in the other treatments (Table S4). After nine generations significant differences between the treatments were found in plant height,

petal length and flower diameter (Table S5). A significant interaction between treatment x replicate was found in the number of flowers but also in petal length, petal width, plant height and the amount of nectar produced per flower, showing that treatment differences in these traits varied strongly among the replicates (Table S5). Discriminant function analysis of the flower morphology in the ninth generation revealed that the three treatments differed significantly from each other (Fig 2C, function 1-2, Wilk's lambda = 0.728, $\chi_8^2 = 99.884$, $p < 0.001$; function 2, Wilk's lambda = 0.880, $\chi_3^2 = 40.066$, $p < 0.001$). The sugar quantity, more precisely the total amount of sugar produced per flower, did not differ between the first and the ninth generation, and also not between the treatments in the ninth generation (Fig S2A, $F_{3,153} = 1.237$, $p = 0.298$).

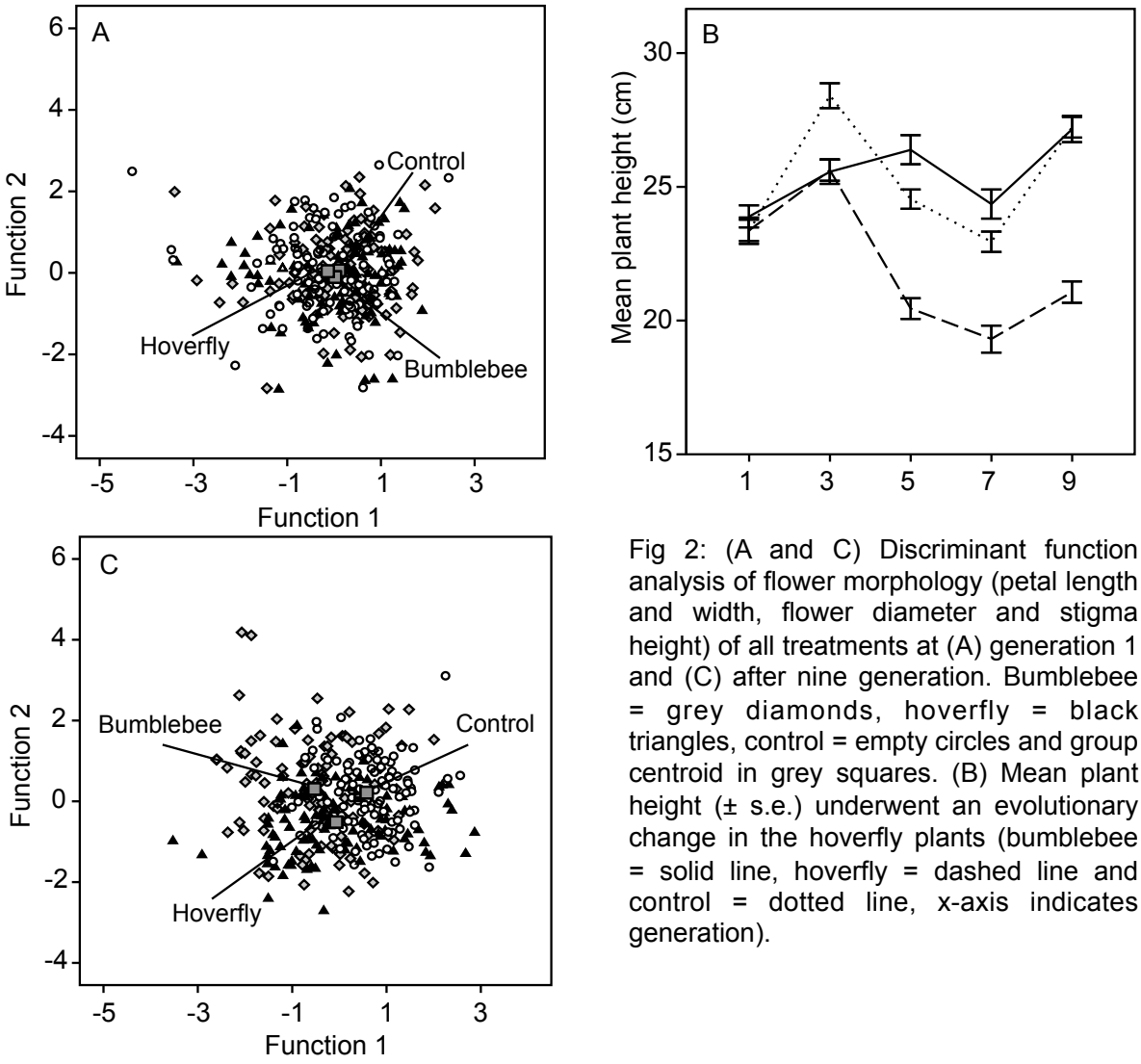

Fig 2: (A and C) Discriminant function analysis of flower morphology (petal length and width, flower diameter and stigma height) of all treatments at (A) generation 1 and (C) after nine generation. Bumblebee = grey diamonds, hoverfly = black triangles, control = empty circles and group centroid in grey squares. (B) Mean plant height (± s.e.) underwent an evolutionary change in the hoverfly plants (bumblebee = solid line, hoverfly = dashed line and control = dotted line, x-axis indicates generation).

During our experiment also floral scent compounds underwent evolutionary changes in the treatments (Table 1). The total amount of scent produced per flower was

found to undergo evolutionary changes most pronouncedly in the bumblebee plants where it increased over time and less pronouncedly in the hoverfly plants (Table S4). In the nitrogen-containing aromatics, we found that methyl anthranilate, indole and benzyl nitrile increased in the bumblebee treatment, while no changes occurred in the hoverfly and control treatment (Fig 3A, Table S4). An exception was 2-aminobenzaldehyde, which remained unchanged in bumblebee plants but decreased in hoverfly and control plants (Table S4). Within the aromatics, benzaldehyde and methyl benzoate were found to increase in all three treatments while phenylacetaldehyde was found to decrease in the hoverfly treatment (Table S4). *P*-anisaldehyde was found to increase in the bumblebee treatment but not in the other treatments (Fig 3B, Table S4). Increments in bumblebee and hoverfly treatments were also found in the fatty acid derivate (*Z*)-3-hexenyl acetate, which did not undergo evolutionary changes in the control treatment (Table S4). Within floral scent, after nine generations significant differences between the treatments were found in *p*-anisaldehyde and methyl anthranilate, which were emitted in the highest amount in the bumblebee treatment (Table S5). The interaction treatment x replicate was found to be significant in all scent compounds except methyl salicylate, methyl anthranilate and 1-butene-4-isothiocyanate, indicating that in these compounds differences between the treatments varied strongly among the replicates (Table S5). A discriminant function analysis of the scent bouquets from the first and ninth generation revealed that all the treatments differed clearly from each other, but the control treatment being closer to the first generation than the other two treatments (Fig 3C, function 1-3,Wilk's lambda =0.553, χ_{45}^2=243.887, p < 0.001; function 2-3, Wilk's lambda=0.741, χ_{28}^2=123.404, p<0.001;function 3, Wilk's lambda =0.934, χ_{13}^2=27.922, p = 0.006).

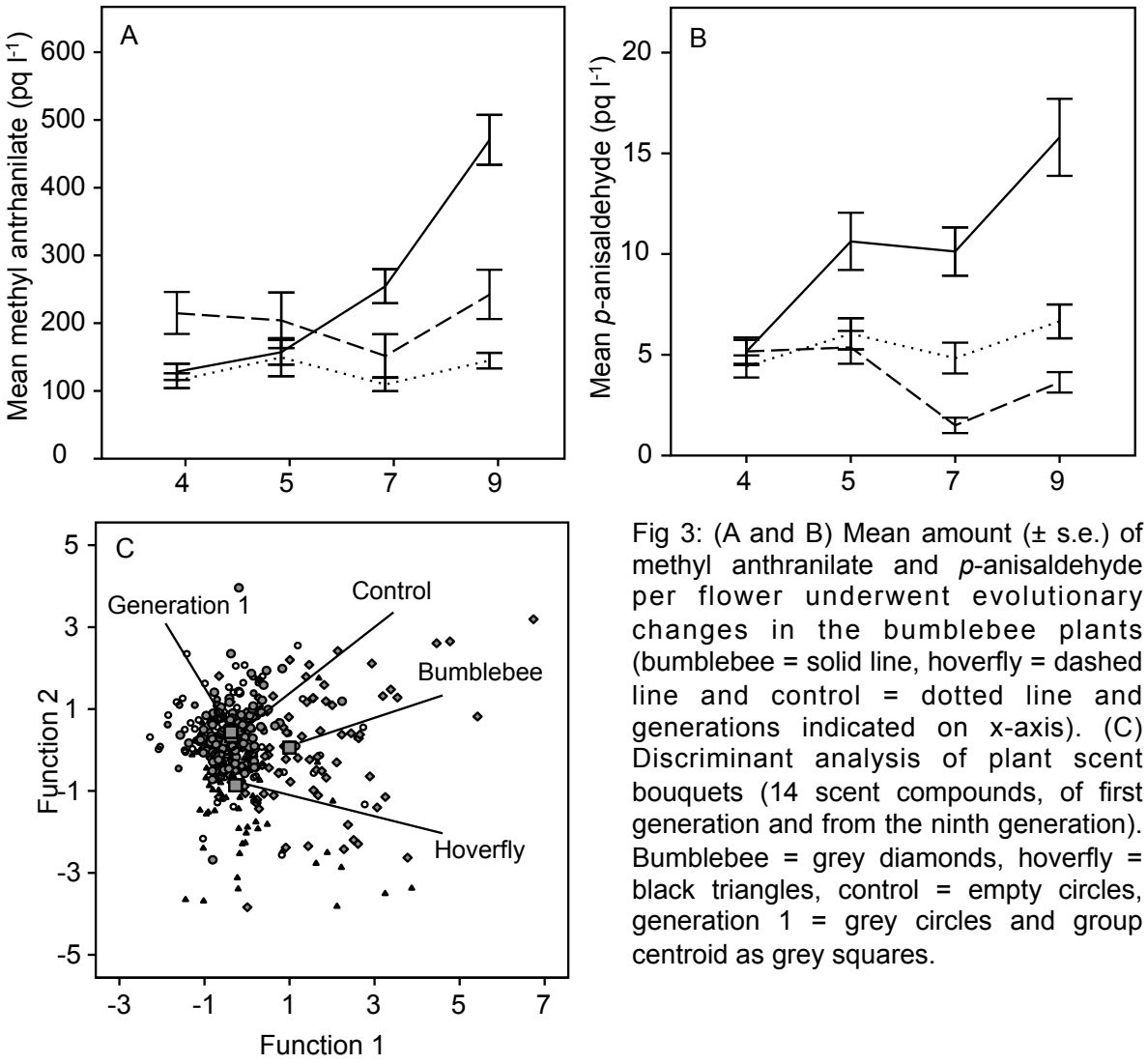

Fig 3: (A and B) Mean amount (± s.e.) of methyl anthranilate and *p*-anisaldehyde per flower underwent evolutionary changes in the bumblebee plants (bumblebee = solid line, hoverfly = dashed line and control = dotted line and generations indicated on x-axis). (C) Discriminant analysis of plant scent bouquets (14 scent compounds, of first generation and from the ninth generation). Bumblebee = grey diamonds, hoverfly = black triangles, control = empty circles, generation 1 = grey circles and group centroid as grey squares.

Floral color was also found to have undergone evolutionary changes over time (Table 1). The PC factor comprising the reflectance values from 290-400nm decreased in the hoverfly and control treatment during the nine generations while remained constant in the bumblebee treatment (Table S4). Positive increments in the reflectance values from 410-490nm were found in the bumblebee and control treatment and were unchanged in the hoverfly group (Tables S4). Additionally, reflectance values from 260-280nm increased in all treatments (Table S4). After nine generations all color PC factors were found to be significant for the interaction treatment x replicate, indicating that treatment differences varied strongly among the replicates in these traits (Table S5).

In regard to defense traits, the total amount of glucosinolates showed a trend to decrease in all treatments but only in the hoverfly treatment the decrease was found to be significant (Fig S2B, $t_{23} = 2.784$, p = 0.011). Nevertheless there was no difference in

the total amount of glucosinolates in the ninth generation between the treatments ($F_{2,34}$ = 1.742, p = 0.190).

Pollination efficiency and fecundity

Pollination efficiency (seeds per fruit) and fecundity (seed set per visited plant) were found to undergo evolutionary change during the nine generations in the hoverfly treatment (Table 1). In the first generation pollination efficiency differed significantly between all three treatments (Table S3). More precisely, hand pollinated plants showed the highest number of seeds per fruit followed by bumblebees that were much more efficient pollinators than hoverflies. The same was true for fecundity, which differed significantly between bumblebee and hoverfly plants with bumblebee plants producing 16x times more seeds per visited plant (Table S3). Evolutionary changes of were found to occur in pollination efficiency sand fecundity. Pollination efficiency was found to increase in the hoverfly treatment within the course of the experiment whereas no change was found in the two other treatments (Fig 4A, Table S4). Similar, fecundity was found to increase in hoverfly plants over time while bumblebee plants showed no changes (Fig S3, Table S4). After nine generations pollination efficiency was found to be still significantly different between the three treatments (Table S5). However, after nine generations fecundity did not differ anymore between hoverfly and bumblebee plants as the fecundity increased 10fold in the hoverfly plants (Table S5). But in both cases the interaction treatment x replicate was significant, indicating that the differences between the treatments in efficiency and fecundity varied strongly among the replicates (Table S5).

Seed quality

The average seed weight and the germination rate were found to differ among the treatments (Fig S4). The average seed weight differed significantly among the treatments with control plants having the highest seed weight, while no differences existed between hoverfly and bumblebee plants (Fig S4A, $F_{2,560}$ = 10.438, p < 0.001). A similar pattern was found in the germination rate where seeds of the control treatment germinated significantly better than the ones from the hoverfly and bumblebee treatment, which showed no differences (Fig S4B, $F_{2,42}$ = 3.391, p = 0.043).

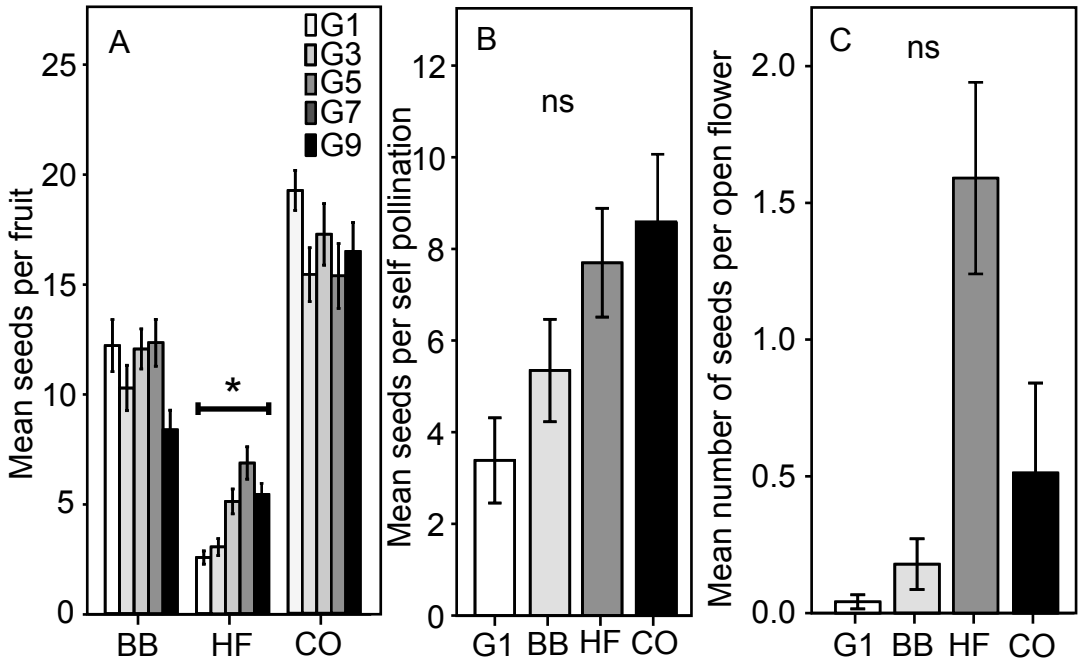

Fig 4: (A) Pollination efficiency of visited plants as mean number of seeds per fruit (± s.e.) of all three treatments during nine generations (CO = control, BB = bumblebee, HF = Hoverfly, G1 - G9 = Generation 1 - Generation 9, N = 678). Asterisks above the bars indicate that evolutionary changes occurred. (B) Self-compatibility as mean number of seeds per self pollination per plant (± s.e.) of first generation plants (G1) and after nine generations. (C) Autogamy as mean number of seeds per open flower (± s.e.) of first generation plants (G1) and after eight generations in the three treatment groups.

Self-compatibility and autonomous-selfing

Self-compatibility tended to increase over time in all treatments (Fig 4B) and the ability for autonomous-selfing was found to increase over time in the hoverfly treatment (Fig 4C), but these changes were found to be strongly variable in the replicates. GLM analysis revealed that self-compatibility was not significant different between the treatments ($F_{3,131}$ = 1.743, p = 0.257), but there was a significant treatment x replicate interaction ($F_{6,131}$ = 2.761, p =0.015), showing that self-compatibility between the treatments differed strongly among the replicates. A similar pattern was found in the ability for autonomous-selfing. While the pollinator treatment had no significant effect on autonomous selfing ($F_{3,81}$ = 2.266, p = 0.181), the interaction treatment x replicate was significant ($F_{6,81}$ = 9.772, p < 0.001), indicating that autonomous-selfing among the treatments varied strongly between the replicates.

Reproductive Isolation

Major differences were found in the preferences of different pollinators. Bumblebees significantly preferred bumblebee-selected plants in their first choices while hoverflies did not show such any preference (Fig S5). Although bumblebees preferred bumblebee-selected plants, floral isolation as estimated by switches between plants of the different treatments was weak (mean isolation index= 0.208, s.e. = ±0.229) and was not significantly different from zero (t_2 = 0.907, p = 0.460). Post-pollination isolation between bumblebee- and hoverfly-selected plants was found to be slightly higher than floral isolation but also not significantly higher than zero (mean isolation index = 0.271, s.e. = ±0.128; t_5 = 2.113, p = 0.088). Post-pollination isolation between replicates within the treatments (mean isolation index = -1.792, s.e. = ±0.778) was found to be significantly lower than between treatments (t_{10} = 2.617, p = 0.026) highlighting that isolation is stronger between treatments than between replicates within treatments. The total reproductive isolation was found to be 0.423 between bumblebee- and hoverfly-selected plants, assuming bumblebees as the only pollinators.

Discussion

The common model of pollinator-driven speciation states that contrasting pollinator environments promote divergence as plants adapt to their co-occurring pollinators (Stebbins 1970; Johnson 2006; Van der Niet and Johnson 2012). But to promote divergence, pollinators should mediate different selective pressures based upon their morphology and preferences on floral traits. The huge numbers of studies on pollinator-mediated selection indeed show huge variations in preferences and selection among pollinators (Galen 1989; Campbell et al. 1997; Fenster et al. 2004; Huber et al. 2005; Benitez-Vieyra et al. 2006; Harder and Johnson 2009). As a consequence of pollinators having varying preferences, a shift in pollinators should cause a change in the selection regime and with it a change in plant-trait evolution. In South Africa it has been shown how a mosaic of long-proboscid flies with different morphologies and preferences has promoted floral divergence (Johnson and Steiner 1997). However, the details of the process of plant trait evolution due to pollinator shifts are still poorly understood, as experimental data is scarce. This study shows to our knowledge for the first time the

evolutionary consequences of pollinator shifts on plant traits in an experimental evolution approach. In our study we found that the pollinators not only differ in the selection they impose but also that after nine generations *B. rapa* plants evolved different phenotypes corresponding to the pollinator environments they have been exposed to.

Evolutionary changes in plant traits and pollinator-mediated selection

In our study, floral scent showed the clearest signature of adaptive evolution. Floral scent plays an important role in several plant-animal interactions acting as attractant or repellent (reviewed in Raguso 2008). However, in phenotypic and selection studies floral scent has often been excluded (but see Schiestl et al. 2011; Ehrlén et al. 2012; Parachnowitsch et al. 2012). In our study, pollinator-mediated selection on floral scent compounds was found to differ between bumblebees and hoverflies especially since bumblebees showed significant selection on multiple scent compounds while hoverflies showed selection on only one scent compound. This is not surprisingly as it is generally assumed that syrphids (Diptera, Syrphidae) use visual cues, especially yellow flower color, to find food sources (Sutherland et al. 1999; Shi et al. 2009). However, it has been shown that the syrphid fly *Episyrphus balteatus* uses olfactory cues for finding non-yellow flowers (Primante and Dötterl 2010). The near absence of selection on floral scent can be explained that hoverflies used in first line the *Brassica* yellow flower color as a cue, for which they are known to have an innate preference (Sutherland et al. 1999). On the other hand bumblebees are known to learn and use floral scent for discriminating plants and finding rewards (Galen et al. 1987; Molet et al. 2009; Suchet et al. 2011; Knauer and Schiestl 2015). Of interest is the aromatic compound *p*-anisaldehyde, which has been shown in GC-EAD (gas chromatography-electroantennographic detection) analysis to elicit responses in the olfactory neurons of *B. terrestris* (Knauer and Schiestl 2015). Additionally, a recent study in *B. rapa* showed for the first time, that the heritability of selected scent compounds ranged between 20 % and 45 % (Zu et al. 2015). This supports our findings that the strong positive selection on *p*-anisaldehyde by bumblebees is responsible for its rise in bumblebee-plants. Bumblebees showed also similar strong positive selection on several other scent compounds, which emission also increased over time. For example, methyl benzoate is known to be the major scent compound in snapdragons, which are pollinated by

bumblebees and may act as an attractant or for discriminating plants (Suchet et al. 2011). But if all compounds under positive selection in our study act generally as attractants in bumblebees is not known. While we do not know if the bumblebees have innate preferences for the scent compounds under positive selection, it is probable that bumblebees selected generally for stronger scented plants, as these are probably easier to detect.

In general the emission of aromatics and nitrogen-containing compounds increased strongly in the bumblebee treatment. This is interesting as most of these compounds were found to be under no direct or even under direct negative selection in the bumblebee treatment. However, analysis of selection differentials reveals, that these compounds are under significant positive selection or under no negative selection anymore. Such differences between selection differentials and gradients indicate correlations among the scent compounds as selection differentials represent direct selection on a trait as well as selection via correlated traits while selection gradients control for correlations (Lande and Arnold 1983). It is therefore most likely that compounds under no direct selection increased due to pleiotropy with the scent compounds that were under strong positive selection. Bumblebees showed strong selection on many morphological traits, which could also have pleiotropic effects on floral scent compounds and vice-versa. Indeed, artificial selection on selected scent compounds in *B. rapa* showed to have not only pleiotropic effects on other scent compounds but also on morphological traits (Zu et al. 2015). So while selection gradients offer us to determine the targets of selection, selection differentials help us to predict evolution much better as evolution is not only driven by direct selection but also by indirect selection trough correlations. These findings show that pleiotropy plays an important role in shaping the scent bouquets. However, as studies on the pleiotropic relations between scent compounds as well as morphological traits still are scarce, more research in this field is needed.

While bumblebees showed strong evolutionary signals in floral scent compounds, hoverfly plants underwent some strong morphological changes. In general, hoverflies do not show a strong preferences for a particular plant species and tend to visit the most abundant and rewarding plants (Branquart and Hemptinne 2000). But in bioassays and field observations it could be shown that the hoverfly *Episyrphus balteatus* preferred yellow, smaller flowers and also exhibited flower constancy (Goulson and Wright 1998; Sutherland et al. 1999). In our study hoverfly-plants decreased strongly in height, which

was surprisingly as hoverflies showed no negative selection for plant height. Instead they showed strong selection differentials for bigger flowers and more importantly for increased plant height (indicating selection via correlations), which was contrary to the evolutionary changes observed. It is possible that there might have been trade-offs with plant traits not measured that caused the reduction in plant height. Alternatively, hoverfly plants showed a trend to increased autonomous-selfing (i.e. spontaneous self-pollination). Selfing plants were shown previously to have less vegetative mass and reduced flowers and higher inbreeding which has negative effects on plant traits as well as fitness (Ornduff 1969; Crnokrak and Roff 1999; Harder and Aizen 2010). In *Mimulus guttatus* it could be shown that in enforced selfing plants, inbreeding caused a strong reduction in flower number and biomass (Dudash et al. 1997). However, the comparison of the germination rates and seed weight in our study rather indicates that if inbreeding depression was present, it was not specifically prevalent in the hoverfly treatment when compared to the bumblebee treatment. This suggests that other factors, which are currently unknown to us, may have influenced the reduction in plant height of the hoverfly plants.

In general, our findings show that bumblebees imposed strong pollinator-mediated selection on plant traits (especially floral scent) while hoverflies showed a rather weaker selection regime. As a consequence evolutionary changes on plant traits were, with few exceptions, more pronounced in bumblebee plants.

Changes in mating-system

The reproductive assurance hypothesis predicts that selection will favor increased self-pollination where pollinator services limit reproduction due to low mate abundance or pollinator scarcity (Baker 1955; Stebbins 1957). Indeed in a pollinator exclusion experiment in *Mimulus guttatus* it was shown that the plants without pollinators evolved an improved ability for self-fertilization (Roels and Kelly 2011). Additionally, in fluctuating pollinator environments it has also been shown that selfing rates increase when pollinator visits are infrequent (Kalisz et al. 2004). Although inbreeding depression is regarded as major force in opposing the evolution of selfing, mixed-mating systems are found to be frequent in angiosperms (reviewed in Goodwillie et al. 2005). Moreover, such mixed-mating populations have been shown to be stable over time (Winn et al. 2011; Wright et al. 2013).

Albeit strongly replicate specific, we found that autonomous-selfing rates showed a trend to increase in the hoverfly treatment. Hoverflies were poor pollinators, as seen in the low fecundity and pollination efficiency of those plants in the first generations. Additionally, pollen flow in hoverfly plants was observed to occur mostly within an individual plant and less between different plants (Daniel Gervasi personal observation). All this suggests that plants visited by hoverflies are under strong pollen limitation. In *Eryismum mediohispanicum* it could be shown that pollen limitation was stronger in populations with low-efficiency pollinators, which is in agreement to our findings (Gomez et al. 2010). Pollen limitation, which is found to be ubiquitous among Angiosperms (Ashman et al. 2004), has been suggested to be an important factor in the evolution of increased selfing rates (Porcher and Lande 2005; Devaux et al. 2014). We think that pollen limitation through reduced pollen transfer, (seen in the pollination efficiency), may have played an essential role in the propagation of autonomous-selfing in the hoverfly treatment. As a consequence of pollen limitation, hoverflies may have indirectly selected for an increased ability to self, explaining the trend to increase in autonomous-selfing in hoverfly plants after nine generations. This switch to a mixed-mating system may also explain why the fecundity and pollination efficiency increased in hoverfly plants. But the replicate-specific effect also suggests that while hoverflies may promote autonomous-selfing, it does not emerge and spread always in the same degree. Also replicate-specific, self-compatibility showed a trend to increase within the hoverfly plants, which is not surprisingly as self-incompatibility is thought to be a major mechanism preventing selfing (Goodwillie et al. 2005; Takayama and Isogai 2005). Interestingly a trend to increase in self-compatibility was also found in control plants, but in autonomous-selfing only weakly, which were pollinated most efficiently among the three treatments and not under pollen limitation. This rather indicates that low gene flow due to absence of multiple paternities may caused the trend in increase in self-compatibility as each control plant, while being pollen donor to more than one plant, receives pollen from only one plant. Thus with ongoing generations the probability rises that plants with identical SI locus will be crossed, promoting the loss of self-incompatibility. In bumblebee plants, we found that autonomous-selfing did not show a trend to increase during the experiment while self-compatibility showed a weak trend to increase. Bumblebees were efficient pollinators and the bumblebee plants also experienced multiple paternities, which allowed for a good genetic mixture countering self-compatibly and autonomous-selfing. Bumblebees visited individual plants on

average 1.36 times and therefore carried probably pollen from several different plants. These findings show how profound and fast pollinator shifts may affect the mating system in plants, especially if the pollinators differ strongly in their pollination behavior.

Reproductive isolation

Prezygotic barriers have been suggested to contribute more to total reproductive isolation in plants than postzygotic barriers and also to evolve faster (Moyle et al. 2004; Rieseberg and Willis 2007; Lowry et al. 2008). But the relative strength of such barriers may differ between plant species and depend on their pollination systems (Cozzolino and Scopece 2008). In our study we found that no floral isolation evolved between bumblebee and hoverfly plants. Although bumblebees preferred their "own plants" over hoverfly plants, floral isolation was found to be slightly weaker compared to post-pollination isolation which evolved faster. Post-pollination isolation was found to be stronger between the pollinator treatments than between the replicates within a treatment. This excludes lineage effects as cause for the post-pollination isolation and indicates that the different pollinator treatments were the cause for increased isolation. As we do not know if the isolation occurs before or after (or in both stadiums) the formation of the zygote it is unclear if prezygotic post-pollination or postzygotic isolation evolves faster. However in three angiosperm genera it was found that there was no difference in the speed of evolution between prezygotic post-pollination isolation and postzygotic isolation (Moyle et al. 2004). Floral isolation is suggested to play an important role in specialized pollination systems while in more generalized pollination system later acting barriers are thought to play a more important role (Cozzolino and Scopece 2008; Schiestl and Schluter 2009). This could explain why floral isolation did not evolve faster then postzygotic isolation. A possible factor promoting reproductive isolation may be found in the increased selfing rates in hoverfly plants. Selfing plants have been argued to promote prezygotic as well as postzygotic isolation therefore acting as speciation booster (reviewed in Wright et al. 2013). Given more time in allopatry it could be possible that the reproductive barriers could become strong enough to prevent the blending of the two plant groups.

Final remarks

Climate change, habitat fragmentation and invasive species can cause shifts of pollinator assemblages and disrupt plant-pollinator interactions (Parmesan et al. 1999). It is therefore important to understand the process and the consequences that such shifts in pollinator environments can have on plant trait evolution. This study demonstrates that plant traits evolve quickly in response to new pollinator environments with different selection regimes and these pollinator shifts can have strong effects on plant phenotype, mating system and fecundity. Moreover, it shows the importance floral scent can have in plant-pollinator interactions and should therefore be incorporated in future studies on plant-pollinator interactions. However, while we quantified a multitude of phenotypic traits, the genetic aspect remains largely unknown in our study. It would be therefore the next step to analyze the genetic background of the evolutionary changes observed in our experiment. We encourage other researchers to perform more experimental evolution experiments in other plant-pollinator systems, as this may give us a better understanding of how pollinators have shaped todays floral and plant diversity.

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Supplementary data

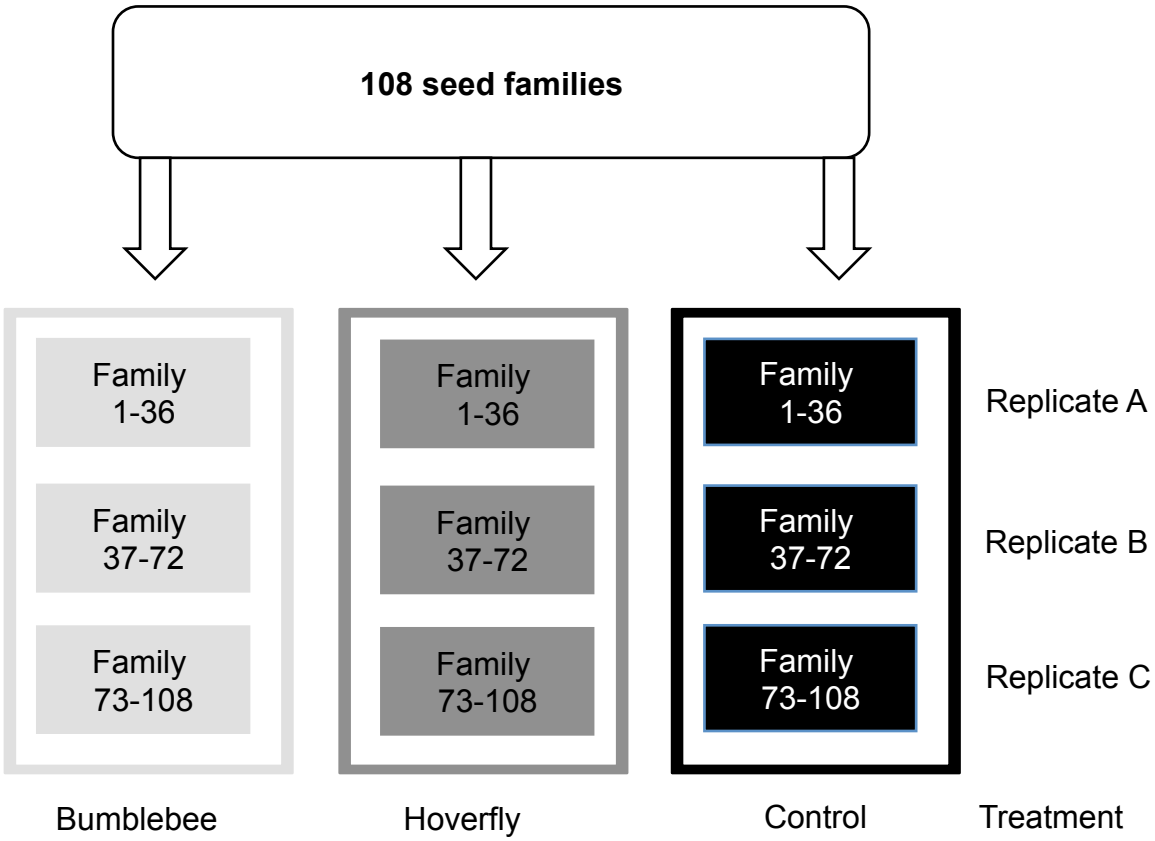

Fig S1: Experimental set-up of the different pollinator treatments at the first generation stage. Each family in every replicate is represented by one plant.

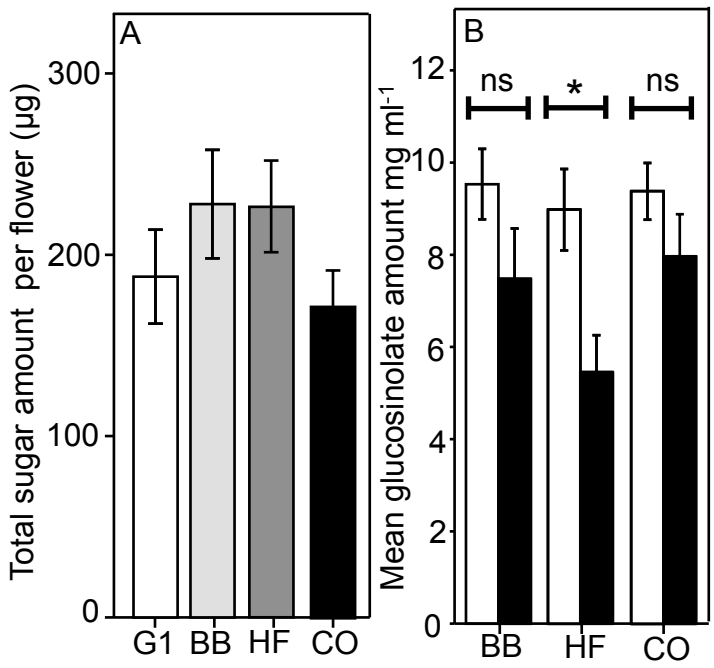

Fig S2: (A) Mean (± s.e.) amount of sugar per flower of first generation plants (G1) and after nine generation (ANOVA, $p = 0.298$). (B) Mean amount of glucosinolate (± s.e.) in all three treatments (CO = control, BB = bumblebee, HF = hoverfly) of the first (white bars) and ninth generation (black bars). Asterisks above the bar indicate significant differences between the generations (*t*-test, $p < 0.05$).

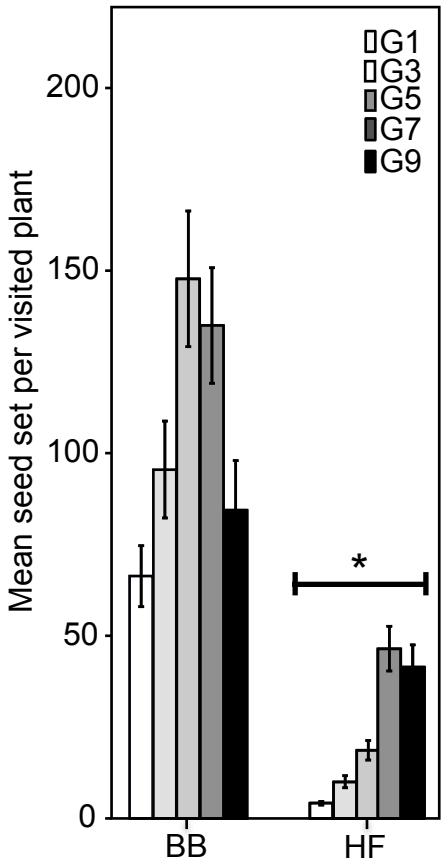

Fig S3: Mean reproductive success (± s.e.) as seed set per visited plant (BB = bumblebee, HF = hoverfly, G1 - G9 = Generation 1 - Generation 9, N = 464). Asterisks above the bars indicate that evolutionary changes occurred.

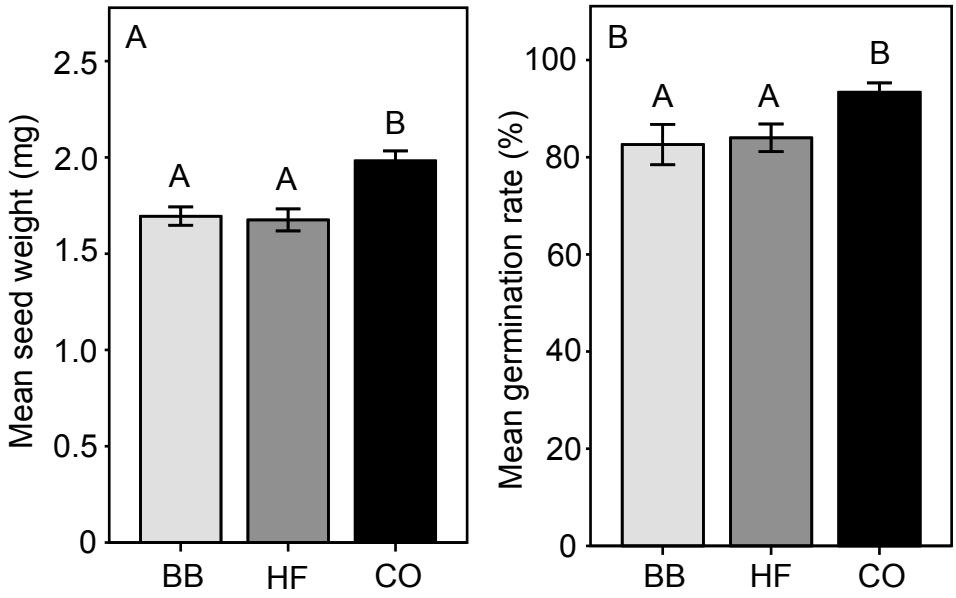

Fig S4: (A) Mean seed weight (± s.e.) of visited plants, and (B) mean replicate germination rate (± s.e.) of each treatment (BB = bumblebee, HF = hoverfly, CO = control) over all replicates and generations combined. Different letters above the bar indicate significant differences (LSD *post-hoc*, $p < 0.05$)

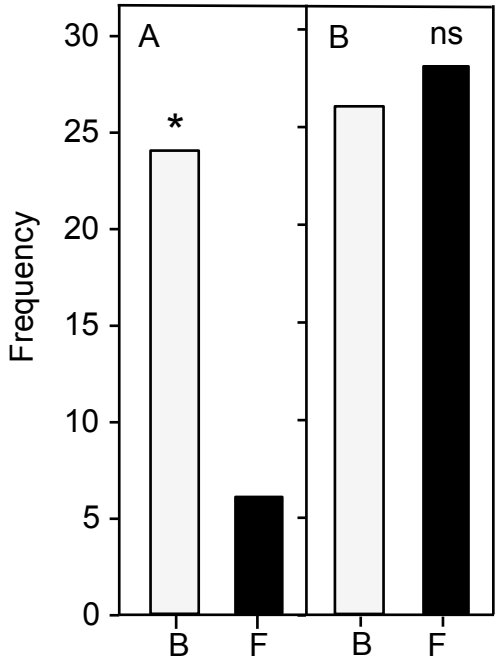

Fig S5: First choices of (A) bumblebees (N = 30) and (B) hoverflies (N = 56) in experiments when allowed to feed on 36 plants (B = bumblebee plants, F = hoverfly plants). Asterisks above the bar represent significant differences to the test value (Binomial test, test-prop = 0.5, $p < 0.05$).

Table S1: Pollinator-mediated selection differentials (S) and gradients (β) with standard error (± s.e.) of 25 plant traits for each pollinator treatment based on a generalized linear model with poisson distribution. Number of visits was used as the fitness variable. Numbers in bold indicate significant selection based on univarate (S) or multivariate (β) generalized linear model.

	Bumblebee								Hoverfly								Control							
Trait	**S ± s.e.**	**df**	**Wald X^2**	**Sig.**	**β ± s.e.**	**df**	**Wald X^2**	**Sig.**	**S ± s.e.**	**df**	**Wald X^2**	**Sig.**	**β ± s.e.**	**df**	**Wald X^2**	**Sig.**	**S ± s.e.**	**df**	**Wald X^2**	**Sig.**	**β ± s.e.**	**df**	**Wald X^2**	**Sig.**
Morphology and nectar																								
Flower number	0.503 ± 0.057	1	77.080	**<0.001**	0.390 ± 0.067	1	33.579	**<0.001**	0.268 ± 0.058	1	21.203	**<0.001**	0.209 ± 0.067	1	9.827	**0.002**	0.161 ± 0.072	1	5.036	**0.025**	0.147 ± 0.076	1	3.701	0.054
Stigma heigth	0.083 ± 0.064	1	1.675	0.196	-0.072 ± 0.072	1	0.989	0.320	0.034 ± 0.064	1	0.286	0.593	-0.006 ± 0.067	1	0.008	0.928	0.053 ± 0.076	1	0.482	0.487	0.001 ± 0.081	1	0.000	0.989
Petal lenght	0.215 ± 0.064	1	11.238	**0.001**	0.260 ± 0.103	1	6.390	**0.011**	0.173 ± 0.065	1	7.153	**0.007**	0.063 ± 0.102	1	0.380	0.537	0.164 ± 0.075	1	4.759	**0.029**	0.172 ± 0.108	1	2.537	0.111
Petal width	0.325 ± 0.065	1	25.237	**<0.001**	0.159 ± 0.085	1	3.504	0.061	0.037 ± 0.064	1	0.344	0.557	-0.100 ± 0.075	1	1.777	0.183	0.068 ± 0.076	1	0.804	0.370	-0.038 ± 0.092	1	0.170	0.680
Flower diameter	0.159 ± 0.065	1	5.905	**0.015**	-0.116 ± 0.107	1	1.176	0.278	0.208 ± 0.067	1	9.802	**0.002**	0.144 ± 0.104	1	1.898	0.168	0.116 ± 0.078	1	2.172	0.141	-0.008 ± 0.106	1	0.006	0.937
Plant heigth	0.562 ± 0.064	1	77.255	**<0.001**	0.353 ± 0.077	1	21.181	**<0.001**	0.198 ± 0.064	1	9.508	**0.002**	0.070 ± 0.075	1	0.871	0.351	0.094 ± 0.077	1	1.521	0.218	0.029 ± 0.086	1	0.116	0.733
Nectar per flower	0.120 ± 0.069	1	3.068	0.080	0.013 ± 0.070	1	0.036	0.850	0.125 ± 0.070	1	3.243	0.072	0.094 ± 0.072	1	1.722	0.189	0.097 ± 0.081	1	1.443	0.230	0.090 ± 0.083	1	1.156	0.282
Scent																								
Aromatics																								
Benzaldehyde	-0.027 ± 0.070	1	0.148	0.700	-0.211 ± 0.089	1	5.585	**0.018**	-0.121 ± 0.074	1	2.620	0.105	-0.102 ± 0.098	1	1.074	0.300	0.011 ± 0.085	1	0.017	0.896	-0.064 ± 0.102	1	0.386	0.534
Phenylacetaldehyde	0.100 ± 0.068	1	2.179	0.140	0.233 ± 0.124	1	3.508	0.061	-0.074 ± 0.076	1	0.939	0.333	-0.071 ± 0.121	1	0.344	0.558	0.073 ± 0.083	1	0.761	0.383	0.003 ± 0.150	1	0.001	0.982
Methylbenzoate	0.257 ± 0.071	1	13.226	**<0.001**	0.322 ± 0.105	1	9.379	**0.002**	-0.065 ± 0.075	1	0.765	0.382	-0.071 ± 0.112	1	0.401	0.527	0.072 ± 0.085	1	0.714	0.398	0.085 ± 0.109	1	0.610	0.435
Phenylethyl alcohol	0.005 ± 0.070	1	0.005	0.943	-0.140 ± 0.112	1	1.571	0.210	-0.087 ± 0.076	1	1.293	0.255	-0.027 ± 0.112	1	0.057	0.811	0.082 ± 0.084	1	0.955	0.329	0.018 ± 0.132	1	0.018	0.894
Methyl salicylate	0.085 ± 0.071	1	1.437	0.231	-0.028 ± 0.095	1	0.087	0.768	0.004 ± 0.075	1	0.003	0.954	0.072 ± 0.091	1	0.632	0.427	-0.006 ± 0.085	1	0.006	0.940	-0.064 ± 0.102	1	0.389	0.533
p-Anisaldehyde	0.166 ± 0.069	1	5.759	**0.016**	0.211 ± 0.088	1	5.817	**0.016**	-0.033 ± 0.075	1	0.193	0.661	-0.069 ± 0.090	1	0.589	0.443	0.000 ± 0.085	1	0.000	0.995	-0.057 ± 0.103	1	0.311	0.577
Terpenoids																								
β-Pinene	0.070 ± 0.069	1	1.032	0.310	0.142 ± 0.075	1	3.573	0.059	-0.115 ± 0.077	1	2.215	0.137	-0.075 ± 0.088	1	0.720	0.396	0.044 ± 0.084	1	0.272	0.602	0.034 ± 0.092	1	0.137	0.712
α-Farnesene	0.066 ± 0.072	1	0.845	0.358	-0.028 ± 0.087	1	0.100	0.752	0.031 ± 0.075	1	0.175	0.675	0.113 ± 0.092	1	1.519	0.218	0.264 ± 0.091	1	8.487	**0.004**	0.277 ± 0.105	1	6.934	**0.008**
Sulfur-containing compounds																								
1-Butene-4-isothiocyanate	-0.145 ± 0.072	1	4.019	**0.045**	-0.116 ± 0.076	1	2.337	0.126	-0.055 ± 0.076	1	0.429	0.513	0.004 ± 0.082	1	0.002	0.965	0.089 ± 0.083	1	1.134	0.287	0.077 ± 0.089	1	0.757	0.384
Nitrogen-containing aromatics																								
Benzyl nitrile	0.035 ± 0.070	1	0.254	0.614	-0.392 ± 0.145	1	7.315	**0.007**	-0.001 ± 0.074	1	0.000	0.986	0.108 ± 0.115	1	0.883	0.347	0.078 ± 0.085	1	0.834	0.361	-0.066 ± 0.167	1	0.157	0.692
2-Aminobenzaldehyde	0.063 ± 0.072	1	0.757	0.384	0.105 ± 0.117	1	0.799	0.372	0.089 ± 0.077	1	1.330	0.249	0.027 ± 0.111	1	0.060	0.807	0.120 ± 0.089	1	1.811	0.178	0.178 ± 0.151	1	1.394	0.238
Indole	0.174 ± 0.072	1	5.759	**0.016**	0.297 ± 0.135	1	4.818	**0.028**	0.034 ± 0.075	1	0.204	0.652	0.106 ± 0.119	1	0.800	0.371	0.098 ± 0.087	1	1.287	0.257	-0.059 ± 0.144	1	0.167	0.683
Methyl anthranilate	0.182 ± 0.076	1	5.813	**0.016**	-0.071 ± 0.126	1	0.319	0.572	-0.036 ± 0.074	1	0.238	0.626	-0.174 ± 0.123	1	2.014	0.156	0.083 ± 0.086	1	0.931	0.335	-0.014 ± 0.127	1	0.012	0.912
Fatty-acid derivates																								
(Z)-3-Hexenyl acetate	-0.034 ± 0.070	1	0.239	0.625	-0.024 ± 0.080	1	0.092	0.762	-0.192 ± 0.081	1	5.580	**0.018**	-0.156 ± 0.095	1	2.695	0.101	0.019 ± 0.084	1	0.050	0.824	-0.022 ± 0.095	1	0.053	0.817
Color																								
PC1 (510-650nm)	-0.056 ± 0.062	1	0.829	0.363	-0.063 ± 0.063	1	1.011	0.315	0.061 ± 0.063	1	0.956	0.328	0.061 ± 0.022	1	0.961	0.327	0.100 ± 0.075	1	1.792	0.181	0.097 ± 0.075	1	1.696	0.193
PC2 (290-400nm)	0.063 ± 0.063	1	0.984	0.321	0.067 ± 0.065	1	1.064	0.302	0.005 ± 0.064	1	0.007	0.931	0.014 ± 0.064	1	0.048	0.827	-0.114 ± 0.074	1	2.363	0.124	-0.112 ± 0.074	1	2.308	0.129
PC3 (410-500nm)	-0.065 ± 0.064	1	1.032	0.310	-0.064 ± 0.064	1	0.988	0.320	0.033 ± 0.061	1	0.299	0.584	0.037 ± 0.061	1	0.365	0.546	-0.001 ± 0.077	1	0.000	0.991	-0.004 ± 0.076	1	0.003	0.953
PC4 (260-280nm)	0.029 ± 0.060	1	0.233	0.629	0.037 ± 0.061	1	0.363	0.547	-0.106 ± 0.066	1	2.536	0.111	-0.106 ± 0.067	1	2.515	0.113	-0.030 ± 0.076	1	0.161	0.688	-0.024 ± 0.076	1	0.104	0.747

Table S2: Comparison of selection gradients between the bumblebee and hoverfly treatment for each trait-group. Numbers in bold indicate a signifcant interaction between treatment and floral trait (multivariate glm).

Traits	β ± s.e.	Wald X^2	df	Sig.	Traits	β ± s.e.	Wald X^2	df	Sig.
Morphology and nectar					**Scent**				
(Intercept)	-0.749 ± 0.066	128.518	1	<0.001	(Intercept)	-0.764 ± 0.076	100.234	1	<0.001
Treatment	-0.226 ± 0.102	4.926	1	0.026	Treatment	-0.003 ± 0.107	0.001	1	0.976
Flower number	0.209 ± 0.067	9.827	1	0.002	Benzaldehyde	-0.102 ± 0.098	1.074	1	0.300
Stigma heigth	-0.006 ± 0.067	0.008	1	0.928	Phenylacetaldehyde	-0.071 ± 0.121	0.344	1	0.558
Petal lenght	0.063 ± 0.102	0.380	1	0.537	Methylbenzoate	-0.071 ± 0.112	0.401	1	0.527
Petal width	-0.100 ± 0.075	1.777	1	0.183	Phenylethyl alcohol	-0.027 ± 0.112	0.057	1	0.811
Flower diameter	0.144 ± 0.104	1.898	1	0.168	Methyl salicylate	0.072 ± 0.091	0.632	1	0.427
Plant heigth	0.070 ± 0.075	0.871	1	0.351	p-Anisaldehyde	-0.069 ± 0.090	0.589	1	0.443
Nectar per flower	0.094 ± 0.072	1.722	1	0.189	β-Pinene	-0.075 ± 0.088	0.720	1	0.396
Treatment*Flower number	0.181 ± 0.095	3.629	1	0.057	α-Farnesene	0.113 ± 0.092	1.519	1	0.218
Treatment*Stigma height	-0.066 ± 0.098	0.449	1	0.503	1-Butene-4-isothiocyanate	0.004 ± 0.082	0.002	1	0.965
Treatment*Petal length	0.197 ± 0.145	1.841	1	0.175	Benzyl nitrile	-0.156 ± 0.095	2.695	1	0.101
Treatment*Petal width	0.259 ± 0.113	5.225	1	**0.022**	2-Aminobenzaldehyde	0.108 ± 0.115	0.883	1	0.347
Treatment*Flower diameter	-0.260 ± 0.149	3.022	1	0.082	Indole	0.027 ± 0.111	0.060	1	0.807
Treatment*Plant height	0.283 ± 0.107	6.998	1	**0.008**	Methyl anthranilate	0.106 ± 0.119	0.800	1	0.371
Treatment*Nectar per flower	-0.081 ± 0.100	0.652	1	0.419	(*Z*)-3-Hexenyl acetate	-0.174 ± 0.123	2.014	1	0.156
Color					Treatment*Benzaldehyde	-0.109 ± 0.133	0.677	1	0.411
(Intercept)	-0.687 ± 0.063	118.528	1	<0.001	Treatment*Phenylacetaldehyde	0.303 ± 0.173	3.066	1	0.080
Treatment	-0.050 ± 0.089	0.308	1	0.579	Treatment*Methyl benzoate	0.392 ± 0.153	6.546	1	**0.011**
PC1 (510-650nm)	0.061 ± 0.062	0.961	1	0.327	Treatment*Phenylethyl alcohol	-0.133 ± 0.159	0.509	1	0.476
PC2 (290-400nm)	0.014 ± 0.064	0.048	1	0.827	Treatment*Methyl salicylate	-0.100 ± 0.132	0.581	1	0.446
PC3 (410-500nm)	0.037 ± 0.061	0.365	1	0.546	Treatment*p-Anisaldehyde	0.281 ± 0.126	4.975	1	**0.026**
PC4 (260-280nm)	-0.106 ± 0.067	2.515	1	0.113	Treatment*β-Pinene	0.217 ± 0.116	3.513	1	0.061
Treatment*PC1 (510-650nm)	-0.124 ± 0.089	1.971	1	0.160	Treatment*α-Farnesene	-0.141 ± 0.127	1.234	1	0.267
Treatment*PC2 (290-400nm)	0.053 ± 0.091	0.338	1	0.561	Treatment*1-Butene-4-isothiocyanate	-0.119 ± 0.111	1.147	1	0.284
Treatment*PC3 (410-500nm)	-0.100 ± 0.088	1.292	1	0.256	Treatment*Benzyl nitrile	-0.500 ± 0.185	7.300	1	**0.007**
Treatment*PC4 (260-280nm)	0.142 ± 0.090	2.488	1	0.115	Treatment*2-Aminobenzaldehyde	0.078 ± 0.161	0.232	1	0.630
					Treatment*Indole	0.191 ± 0.180	1.120	1	0.290
					Treatment*Methyl anthranilate	0.103 ± 0.176	0.340	1	0.560
					Treatment*(Z)-3-Hexenyl acetate	0.131 ± 0.124	1.116	1	0.291

Table S3: Comparison of plant traits in the first generation between the three treatments. Factors or interactions that have a significant effect on the plant trait are shown in bold (univariate general linear model). Different superscripts indicate significant differences among the treatment groups (Bonferroni *post-hoc*, $p < 0.05$).

Trait		Mean ± s.e.	Factors	df	Type III SOS	F	Sig.
Morphology and nectar							
Flower number	Bumblebee	9.093 ± 0.288	Treatment	2	34.8	0.800	0.510
	Hoverfly	8.741 ± 0.281	Replicate	2	45	1.035	0.434
	Control	9.551 ± 0.367	Treatment*Replicate	4	87	2.095	0.081
Stigma heigth (cm)	Bumblebee	0.466 ± 0.008	Treatment	2	1.2	0.442	0.671
	Hoverfly	0.450 ± 0.008	Replicate	2	2.7	0.968	0.454
	Control	0.460 ± 0.009	Treatment*Replicate	4	5.6	1.945	0.103
Petal length (cm)	Bumblebee	0.609 ± 0.005	Treatment	2	0	0.178	0.843
	Hoverfly	0.607 ± 0.005	Replicate	2	0	0.461	0.660
	Control	0.612 ± 0.005	Treatment*Replicate	4	0	1.392	0.237
Petal width (cm)	Bumblebee	0.517 ± 0.006	Treatment	2	0	0.078	0.927
	Hoverfly	0.512 ± 0.005	Replicate	2	0	1.462	0.334
	Control	0.525 ± 0.012	**Treatment*Replicate**	4	0	2.798	**0.026**
Flower diameter (cm)	Bumblebee	1.365 ± 0.014	Treatment	2	0	0.315	0.747
	Hoverfly	1.352 ± 0.013	Replicate	2	0.2	3.964	0.112
	Control	1.347 ± 0.014	Treatment*Replicate	4	0.1	1.413	0.230
Plant heigth (cm)	Bumblebee	23.894 ± 0.418	Treatment	2	20.6	0.098	0.909
	Hoverfly	23.375 ± 0.396	Replicate	2	142.3	0.677	0.558
	Control	23.358 ± 0.492	**Treatment*Replicate**	4	420.3	5.506	**<0.001**
Nectar per flower (nl)	Bumblebee	73.096 ± 3.795	Treatment	2	1.2	5.959	0.063
	Hoverfly	71.839 ± 4.053	Replicate	2	0.4	2.257	0.221
	Control	71.329 ± 4.448	Treatment*Replicate	4	0.4	0.158	0.959
Color							
PC1 (500-650nm)	Bumblebee	0.075 ± 0.074 a	**Treatment**	2	33.3	10.239	**0.027**
	Hoverfly	-0.169 ± 0.079 b	Replicate	2	2.3	0.693	0.551
	Control	-0.695 ± 0.084 c	**Treatment*Replicate**	4	6.5	2.485	**0.044**
PC2 (290-400nm)	Bumblebee	0.579 ± 0.071	Treatment	2	3.7	2.285	0.218
	Hoverfly	0.565 ± 0.065	Replicate	2	3.1	1.908	0.262
	Control	0.344 ± 0.061	Treatment*Replicate	4	3.3	1.806	0.127
PC3 (410-490nm)	Bumblebee	-0.023 ± 0.080	Treatment	2	7.6	2.401	0.207
	Hoverfly	0.039 ± 0.086	Replicate	2	2.7	0.853	0.491
	Control	-0.313 ± 0.087	Treatment*Replicate	4	6.3	2.100	0.081
PC4 (260-280nm)	Bumblebee	-0.674 ± 0.047 a	**Treatment**	2	16.7	145.816	**<0.001**
	Hoverfly	-0.830 ± 0.045 b	**Replicate**	2	1.1	9.456	**0.030**
	Control	-1.216 ± 0.048 c	Treatment*Replicate	4	0.2	0.244	0.913
Seed production							
Seeds per fruit	Bumblebee	12.189 ± 1.180 a	**Treatment**	2	5176.7	83.008	**0.001**
	Hoverfly	2.631 ± 0.295 b	Replicate	2	117.6	1.886	0.265
	Control	19.231 ± 0.910 c	Treatment*Replicate	4	124.7	1.132	0.346
Seeds per visited plant	Bumblebee	66.289 ± 8.333	**Treatment**	1	73682.2	4056.737	**<0.001**
	Hoverfly	4.282 ± 0.463	Replicate	2	12.5	0.363	0.734
			Treatment*Replicate	2	34.6	0.013	0.988

Table S4: Univariate linear regression analysis with plant trait as dependent variable and generation as the independent variable for each treatment and replicate. Replicates with significant regressions in a plant trait are shown in bold.

Trait	Treatment	Replicate	Coefficient ± s.e.	R^2	t	Sig.
Morphology and nectar						
Flower number	Bumblebee	A	0.103 ± 0.116	.005	.890	.375
		B	**0.325 ± 0.127**	.036	2.550	**.012**
		C	**0.663 ± 0.127**	.134	5.243	**<0.001**
	Hoverfly	A	0.101 ± 0.120	.004	.846	.399
		B	0.219 ± 0.127	.017	1.728	.086
		C	0.094 ± 0.107	.005	.883	.378
	Control	A	0.119 ± 0.113	.006	1.047	.297
		B	0.189 ± 0.111	.016	1.692	.092
		C	-0.091 ± 0.121	.003	-.757	.450
Stigma heigth	Bumblebee	**A**	**-0.202 ± 0.025**	.273	-7.959	**<0.001**
		B	0.036 ± 0.027	.010	1.342	.181
		C	0.018 ± 0.025	.003	.742	.459
	Hoverfly	**A**	**-0.192 ± 0.023**	.305	-8.454	**<0.001**
		B	-0.031 ± 0.025	.009	-1.272	.205
		C	0.040 ± 0.024	.016	1.678	.095
	Control	**A**	**0.079 ± 0.027**	.048	2.988	**.003**
		B	-0.041 ± 0.023	.017	-1.731	.085
		C	-0.018 ± 0.021	.004	-.856	.393
Petal length	Bumblebee	**A**	**-0.006 ± 0.002**	.060	-3.286	**.001**
		B	0.000 ± 0.002	.000	.139	.890
		C	0.001 ± 0.002	.005	.895	.372
	Hoverfly	**A**	**-0.006 ± 0.002**	.075	-3.622	**<0.001**
		B	-0.002 ± 0.002	.005	-.906	.366
		C	**0.004 ± 0.001**	.042	2.723	**.007**
	Control	**A**	**0.004 ± 0.001**	.032	2.425	**.016**
		B	**0.003 ± 0.001**	.024	2.080	**.039**
		C	**0.008 ± 0.002**	.130	5.135	**<0.001**
Petal width	Bumblebee	**A**	**0.007 ± 0.002**	.081	3.860	**<0.001**
		B	-0.002 ± 0.002	.005	-.917	.360
		C	0.002 ± 0.002	.010	1.307	.193
	Hoverfly	**A**	**-0.005 ± 0.002**	.069	-3.475	**.001**
		B	0.000 ± 0.002	.000	.222	.825
		C	**-0.012 ± 0.002**	.240	-7.313	**<0.001**
	Control	A	0.003 ± 0.002	.019	1.835	.068
		B	**-0.004 ± 0.001**	.055	-3.204	**.002**
		C	-0.002 ± 0.002	.008	-1.216	.226
Flower diameter	Bumblebee	A	-0.003 ± 0.004	.004	-.849	.397
		B	**0.010 ± 0.004**	.032	2.383	**.018**
		C	0.000 ± 0.004	.000	.035	.972
	Hoverfly	A	-0.005 ± 0.004	.011	-1.356	.177
		B	-0.002 ± 0.004	.001	-.442	.659
		C	-0.003 ± 0.003	.006	-.977	.330
	Control	A	0.006 ± 0.004	.013	1.504	.134
		B	**0.009 ± 0.003**	.043	2.824	**.005**
		C	**0.011 ± 0.004**	.048	2.982	**.003**
Plant heigth	Bumblebee	A	0.106 ± 0.126	.004	.841	.402
		B	**0.663 ± 0.138**	.119	4.816	**<0.001**
		C	0.047 ± 0.123	.001	.383	.702
	Hoverfly	**A**	**-0.708 ± 0.103**	.154	-5.456	**<0.001**
		B	**-0.580 ± 0.109**	.141	-5.305	**<0.001**
		C	**-0.317 ± 0.117**	.041	-2.703	**.008**
	Control	**A**	**0.600 ± 0.132**	.104	4.544	**<0.001**
		B	0.175 ± 0.115	.013	1.517	.131
		C	**-0.426 ± 0.125**	.062	-3.421	**.001**
Nectar per flower	Bumblebee	**A**	**-0.094 ± 0.037**	.037	-2.536	**.012**
		B	**-0.121 ± 0.044**	.043	-2.778	**.006**
		C	**0.116 ± 0.023**	.121	4.942	**<0.001**
	Hoverfly	**A**	**0.093 ± 0.029**	.058	3.178	**.002**
		B	**0.046 ± 0.023**	.022	1.972	**.050**
		C	0.054 ± 0.032	.017	1.694	.092
	Control	A	-0.004 ± 0.033	.000	-.137	.891
		B	**0.095 ± 0.017**	.148	5.506	**<0.001**
		C	0.039 ± 0.029	.010	1.320	.189

Floral Scent						
Aromatics						
Benzaldehyde	Bumblebee	**A**	**0.135 ± 0.023**	.206	5.823	**<0.001**
		B	**0.249 ± 0.026**	.394	9.438	**<0.001**
		C	**0.302 ± 0.024**	.530	12.556	**<0.001**
	Hoverfly	**A**	**0.194 ± 0.028**	.286	6.960	**<0.001**
		B	**0.133 ± 0.024**	.201	5.631	**<0.001**
		C	**0.266 ± 0.026**	.452	10.402	**<0.001**
	Control	**A**	**0.159 ± 0.023**	.259	7.019	**<0.001**
		B	**0.058 ± 0.028**	.030	2.090	**.038**
		C	**0.242 ± 0.033**	.276	7.313	**<0.001**
Phenylacetaldehyde	Bumblebee	A	0.072 ± 0.044	.020	1.646	.102
		B	-0.025 ± 0.060	.001	-.417	.677
		C	**0.203 ± 0.044**	.133	4.629	**<0.001**
	Hoverfly	**A**	**-0.132 ± 0.049**	.056	-2.689	**.008**
		B	**-0.213 ± 0.052**	.118	-4.102	**<0.001**
		C	0.031 ± 0.055	.003	.573	.567
	Control	A	-0.100 ± 0.054	.024	-1.869	.064
		B	-0.073 ± 0.045	.019	-1.631	.105
		C	**-0.133 ± 0.040**	.074	-3.343	**.001**
Methyl benzoate	Bumblebee	**A**	**0.091 ± 0.040**	.039	2.291	**.024**
		B	**0.221 ± 0.031**	.277	7.244	**<0.001**
		C	**0.487 ± 0.045**	.453	10.766	**<0.001**
	Hoverfly	A	0.037 ± 0.038	.008	.977	.330
		B	**0.182 ± 0.042**	.130	4.341	**<0.001**
		C	**0.200 ± 0.038**	.175	5.271	**<0.001**
	Control	A	0.034 ± 0.035	.007	.974	.332
		B	**0.191 ± 0.029**	.236	6.561	**<0.001**
		C	**0.087 ± 0.027**	.070	3.238	**.002**
Phenylethyl alcohol	Bumblebee	A	0.014 ± 0.019	.004	.732	.465
		B	0.033 ± 0.032	.008	1.050	.296
		C	**0.279 ± 0.023**	.504	11.934	**<0.001**
	Hoverfly	**A**	**-0.108 ± 0.033**	.082	-3.278	**.001**
		B	0.003 ± 0.033	.000	.100	.921
		C	**0.157 ± 0.034**	.142	4.656	**<0.001**
	Control	**A**	**-0.055 ± 0.020**	.048	-2.677	**.008**
		B	**0.063 ± 0.020**	.066	3.132	**.002**
		C	**0.198 ± 0.024**	.325	8.204	**<0.001**
Methyl salicylate	Bumblebee	**A**	**0.120 ± 0.051**	.040	2.350	**.020**
		B	0.034 ± 0.037	.006	.906	.366
		C	**0.311 ± 0.039**	.317	8.058	**<0.001**
	Hoverfly	A	0.015 ± 0.038	.001	.388	.699
		B	0.018 ± 0.031	.003	.573	.568
		C	**0.104 ± 0.035**	.064	2.991	**.003**
	Control	A	-0.052 ± 0.042	.011	-1.249	.214
		B	**0.071 ± 0.035**	.028	2.017	**.046**
		C	**0.078 ± 0.036**	.033	2.178	**.031**
p-Anisaldehyde	Bumblebee	**A**	**0.129 ± 0.045**	.060	2.883	**.005**
		B	0.048 ± 0.041	.010	1.186	.238
		C	**0.242 ± 0.046**	.163	5.229	**<0.001**
	Hoverfly	A	-0.049 ± 0.031	.021	-1.602	.112
		B	-0.043 ± 0.030	.017	-1.458	.147
		C	**-0.114 ± 0.042**	.054	-2.726	**.007**
	Control	A	0.008 ± 0.025	.001	.305	.761
		B	0.031 ± 0.040	.004	.767	.444
		C	**0.109 ± 0.043**	.044	2.552	**.012**
Terpenoids						
β-Pinene	Bumblebee	A	0.018 ± 0.019	.008	.997	.321
		B	**0.046 ± 0.017**	.050	2.689	**.008**
		C	**0.042 ± 0.019**	.034	2.216	**.028**
	Hoverfly	A	0.024 ± 0.023	.009	1.037	.302
		B	0.010 ± 0.019	.002	.546	.586
		C	0.001 ± 0.019	.000	.059	.953
	Control	A	0.013 ± 0.013	.007	1.002	.318
		B	-0.001 ± 0.015	.000	-.092	.927
		C	0.023 ± 0.016	.015	1.479	.141

α-Farnesene	Bumblebee	**A**	**0.136 ± 0.030**	.134	4.510	**<0.001**
		B	-0.002 ± 0.062	.000	-.030	.976
		C	0.041 ± 0.045	.006	.895	.372
	Hoverfly	A	0.054 ± 0.038	.016	1.424	.157
		B	0.023 ± 0.025	.007	.921	.359
		C	0.007 ± 0.050	.000	.130	.897
	Control	A	-0.012 ± 0.028	.001	-.431	.667
		B	**0.195 ± 0.040**	.145	4.862	**<0.001**
		C	0.046 ± 0.033	.013	1.371	.173
Sulphur containing compounds						
1-Butene-4-isothiocyanate	Bumblebee	**A**	**0.234 ± 0.038**	.221	6.088	**<0.001**
		B	**0.209 ± 0.036**	.198	5.812	**<0.001**
		C	**0.188 ± 0.042**	.127	4.523	**<0.001**
	Hoverfly	A	0.029 ± 0.058	.002	.494	.622
		B	**0.141 ± 0.042**	.083	3.383	**.001**
		C	**0.224 ± 0.041**	.188	5.508	**<0.001**
	Control	**A**	**0.171 ± 0.038**	.129	4.560	**<0.001**
		B	**0.159 ± 0.038**	.111	4.160	**<0.001**
		C	0.062 ± 0.048	.012	1.282	.202
Nitrogen-containing aromatics						
Benzyl nitrile	Bumblebee	**A**	**0.165 ± 0.035**	.147	4.743	**<0.001**
		B	0.062 ± 0.040	.017	1.524	.130
		C	**0.276 ± 0.041**	.247	6.785	**<0.001**
	Hoverfly	A	-0.044 ± 0.036	.012	-1.220	.225
		B	-0.044 ± 0.045	.008	-.981	.329
		C	**0.081 ± 0.034**	.042	2.387	**.018**
	Control	A	0.009 ± 0.037	.000	.257	.797
		B	-0.015 ± 0.033	.001	-.454	.650
		C	-0.080 ± 0.044	.023	-1.811	.072
2-Aminobenzaldehyde	Bumblebee	**A**	**0.117 ± 0.053**	.036	2.224	**.028**
		B	-0.030 ± 0.074	.001	-.400	.690
		C	0.050 ± 0.079	.003	.635	.526
	Hoverfly	A	-0.044 ± 0.081	.002	-.546	.586
		B	**-0.326 ± 0.086**	.102	-3.773	**<0.001**
		C	**-0.311 ± 0.087**	.089	-3.575	**<0.001**
	Control	**A**	**-0.106 ± 0.049**	.032	-2.170	**.032**
		B	-0.013 ± 0.046	.001	-.296	.768
		C	**-0.193 ± 0.069**	.053	-2.806	**.006**
Indole	Bumblebee	**A**	**0.173 ± 0.041**	.121	4.246	**<0.001**
		B	0.037 ± 0.046	.005	.798	.426
		C	**0.347 ± 0.044**	.306	7.863	**<0.001**
	Hoverfly	**A**	**-0.116 ± 0.048**	.047	-2.432	**.016**
		B	0.073 ± 0.053	.015	1.382	.169
		C	-0.136 ± 0.073	.026	-1.874	.063
	Control	A	0.026 ± 0.031	.005	.832	.407
		B	0.032 ± 0.039	.005	.815	.416
		C	-0.006 ± 0.047	.000	-.127	.899
Methyl anthranilate	Bumblebee	**A**	**0.318 ± 0.073**	.128	4.381	**<0.001**
		B	**0.167 ± 0.055**	.062	3.018	**.003**
		C	**0.392 ± 0.049**	.315	8.030	**<0.001**
	Hoverfly	A	-0.095 ± 0.083	.011	-1.152	.252
		B	**0.169 ± 0.054**	.072	3.132	**.002**
		C	0.028 ± 0.069	.001	.407	.685
	Control	**A**	**-0.143 ± 0.050**	.054	-2.837	**.005**
		B	**0.202 ± 0.051**	.100	3.933	**<0.001**
		C	0.075 ± 0.043	.021	1.741	.084
Fatty-acid derivates						
(*Z*)-3-Hexenyl acetate	Bumblebee	**A**	**0.163 ± 0.036**	.132	4.470	**<0.001**
		B	**0.104 ± 0.032**	.073	3.279	**.001**
		C	**0.073 ± 0.031**	.037	2.320	**.022**
	Hoverfly	A	-0.053 ± 0.041	.014	-1.297	.197
		B	**0.093 ± 0.033**	.059	2.798	**.006**
		C	**0.189 ± 0.031**	.218	6.049	**<0.001**
	Control	A	0.046 ± 0.037	.011	1.261	.209
		B	**0.091 ± 0.036**	.043	2.506	**.013**
		C	0.078 ± 0.041	.025	1.914	.058

Sum scent						
Total amount of scent	Bumblebee	**A**	**0.155 ± 0.024**	0.24	6.361	**<0.001**
		B	**0.066 ± 0.029**	0.04	2.271	**.025**
		C	**0.213 ± 0.027**	0.30	7.771	**<0.001**
	Hoverfly	A	-0.023 ± 0.022	0.01	-1.020	.310
		B	**0.048 ± 0.021**	0.04	2.241	**.027**
		C	**0.057 ± 0.028**	0.03	2.003	**.047**
	Control	A	-0.017 ± 0.023	0.00	-.713	.477
		B	**0.076 ± 0.023**	0.07	3.326	**.001**
		C	0.050 ± 0.028	0.02	1.807	.073
Color						
PC1 (500-650nm)	Bumblebee	**A**	**0.059 ± 0.029**	.023	1.999	**.047**
		B	**-0.083 ± 0.023**	.073	-3.701	**<0.001**
		C	**-0.146 ± 0.027**	.141	-5.387	**<0.001**
	Hoverfly	A	-0.027 ± 0.024	.008	-1.142	.255
		B	0.023 ± 0.021	.007	1.086	.279
		C	**-0.176 ± 0.026**	.215	-6.718	**<0.001**
	Control	**A**	**0.136 ± 0.022**	.180	6.225	**<0.001**
		B	**0.089 ± 0.023**	.077	3.841	**<0.001**
		C	**-0.084 ± 0.026**	.055	-3.213	**.002**
PC2 (290-400nm)	Bumblebee	**A**	**0.063 ± 0.026**	.033	2.398	**.018**
		B	**-0.063 ± 0.025**	.034	-2.486	**.014**
		C	-0.050 ± 0.028	.017	-1.753	.081
	Hoverfly	**A**	**-0.048 ± 0.023**	.027	-2.128	**.035**
		B	**-0.055 ± 0.025**	.029	-2.231	**.027**
		C	**-0.089 ± 0.026**	.065	-3.401	**.001**
	Control	**A**	**-0.066 ± 0.024**	.041	-2.754	**.007**
		B	**-0.063 ± 0.027**	.029	-2.298	**.023**
		C	**-0.077 ± 0.023**	.062	-3.396	**.001**
PC3 (410-490nm)	Bumblebee	**A**	**0.089 ± 0.032**	.044	2.774	**.006**
		B	**0.086 ± 0.028**	.050	3.022	**.003**
		C	-0.019 ± 0.029	.002	-.661	.509
	Hoverfly	**A**	**-0.107 ± 0.022**	.123	-4.777	**<0.001**
		B	0.036 ± 0.022	.017	1.686	.094
		C	**0.120 ± 0.028**	.100	4.293	**<0.001**
	Control	A	0.003 ± 0.023	.000	.143	.887
		B	**0.055 ± 0.026**	.026	2.157	**.032**
		C	**0.056 ± 0.019**	.046	2.919	**.004**
PC4 (260-280nm)	Bumblebee	**A**	**0.177 ± 0.020**	.325	9.030	**<0.001**
		B	**0.178 ± 0.018**	.372	10.116	**<0.001**
		C	**0.212 ± 0.017**	.481	12.812	**<0.001**
	Hoverfly	**A**	**0.209 ± 0.019**	.428	11.034	**<0.001**
		B	**0.325 ± 0.020**	.619	16.587	**<0.001**
		C	**0.200 ± 0.016**	.472	12.157	**<0.001**
	Control	**A**	**0.264 ± 0.022**	.458	12.238	**<0.001**
		B	**0.357 ± 0.018**	.683	19.475	**<0.001**
		C	**0.213 ± 0.018**	.443	11.826	**<0.001**
Seed production						
Seeds per fruit	Bumblebee	A	-0.534 ± 0.291	.053	-1.834	.072
		B	-0.332 ± 0.256	.027	-1.295	.200
		C	-0.017 ± 0.292	.000	-.058	.954
	Hoverfly	**A**	**0.805 ± 0.160**	.282	5.016	**<0.001**
		B	**0.458 ± 0.131**	.163	3.507	**.001**
		C	0.137 ± 0.098	.030	1.392	.169
	Control	A	-0.188 ± 0.374	.004	-.503	.617
		B	-0.141 ± 0.356	.003	-.397	.693
		C	-0.514 ± 0.328	.041	-1.656	.123
Seeds per visited plant	Bumblebee	A	-3.411 ± 3.819	.013	-.893	.375
		B	3.976 ± 4.303	.014	.924	.359
		C	**9.649 ± 3.805**	.093	2.536	**.014**
	Hoverfly	**A**	**8.106 ± 1.077**	.470	7.527	**<0.001**
		B	**5.731 ± 1.177**	.274	4.871	**<0.001**
		C	**2.672 ± 0.872**	.130	3.063	**.003**

Table S5: Comparison of plant traits in the ninth generation between the three treatments. Floral volatiles are shown in ρg*l^{-1}. Factors or interactions that are significant are shown in bold (univariate general linear model). Different superscripts indicate significant differences among the treatment groups (Bonferroni *post-hoc*, $p < 0.05$).

Trait	Treatment	Mean ± s.e.	Factors	df	Type III SOS	F	Sig.
Morphology and nectar							
Flower number	Bumblebee	12.943 ± 0.531	Treatment	2	97.2	0.543	0.619
	Hoverfly	11.575 ± 0.574	Replicate	2	158.7	0.886	0.480
	Control	12.234 ± 0.418	**Treatment*Replicate**	4	358.4	3.364	**0.010**
Stigma heigth (cm)	Bumblebee	0.426 ± 0.013	Treatment	2	24.9	0.457	0.662
	Hoverfly	0.405 ± 0.011	Replicate	2	43.9	0.807	0.508
	Control	0.471 ± 0.009	**Treatment*Replicate**	4	108.8	35.239	**<0.001**
Petal length (cm)	Bumblebee	0.586 ± 0.006^{a}	**Treatment**	2	0.2	11.057	**0.023**
	Hoverfly	0.589 ± 0.007^{a}	Replicate	2	0.1	6.686	0.053
	Control	0.644 ± 0.005^{b}	**Treatment*Replicate**	4	0	3.043	**0.018**
Petal width (cm)	Bumblebee	0.512 ± 0.008	Treatment	2	0.5	2.276	0.219
	Hoverfly	0.456 ± 0.006	Replicate	2	0.1	1.114	0.412
	Control	0.488 ± 0.005	**Treatment*Replicate**	4	0.1	10.121	**<0.001**
Flower diameter (cm)	Bumblebee	1.330 ± 0.012^{a}	**Treatment**	2	0.5	7.942	**0.040**
	Hoverfly	1.312 ± 0.013^{b}	Replicate	2	0.1	2.272	0.219
	Control	1.402 ± 0.013^{c}	Treatment*Replicate	4	0.1	1.815	0.126
Plant heigth(cm)	Bumblebee	27.165 ± 0.488^{a}	**Treatment**	2	2697.6	10.974	**0.024**
	Hoverfly	21.060 ± 0.396^{b}	Replicate	2	227.4	0.925	0.468
	Control	27.237 ± 0.368^{a}	**Treatment*Replicate**	4	491.7	7.092	**<0.001**
Nectar per flower (nl)	Bumblebee	111.961 ± 10.917	Treatment	2	27.5	0.608	0.588
	Hoverfly	126.428 ± 9.593	Replicate	2	11.8	0.261	0.782
	Control	104.548 ± 7.727	**Treatment*Replicate**	4	90.6	13.005	**<0.001**
Floral Scent							
Aromatics							
Benzaldehyde	Bumblebee	438.174 ± 24.134	Treatment	2	0.7	0.105	0.903
	Hoverfly	431.546 ± 22.619	Replicate	2	11	1.570	0.314
	Control	370.530 ± 14.266	**Treatment*Replicate**	4	14	21.058	**<0.001**
Phenylacetaldehyde	Bumblebee	83.405 ± 21.880	Treatment	2	40	1.070	0.424
	Hoverfly	37.359 ± 4.921	Replicate	2	2.4	0.065	0.938
	Control	25.108 ± 4.552	**Treatment*Replicate**	4	74.7	20.084	**<0.001**
Methyl benzoate	Bumblebee	131.811 ± 17.721	Treatment	2	25	1.341	0.358
	Hoverfly	111.706 ± 11.069	Replicate	2	58.9	3.136	0.152
	Control	48.052 ± 3.282	**Treatment*Replicate**	4	37.6	15.724	**<0.001**
Phenylethyl alcohol	Bumblebee	6.669 ± 0.586	Treatment	2	2.9	1.803	0.277
	Hoverfly	5.636 ± 0.463	**Replicate**	2	74.4	46.723	**0.002**
	Control	4.679 ± 0.308	**Treatment*Replicate**	4	3.2	4.402	**0.002**
Methyl salicylate	Bumblebee	38.792 ± 3.473	Treatment	2	2.9	2.049	0.244
	Hoverfly	28.715 ± 2.530	**Replicate**	2	51.2	35.746	**0.003**
	Control	32.338 ± 3.130	Treatment*Replicate	4	2.9	0.931	0.446
p-Anisaldehyde	Bumblebee	15.792 ± 1.914^{a}	**Treatment**	2	54.7	10.377	**0.026**
	Hoverfly	3.630 ± 0.508^{b}	Replicate	2	11.4	2.165	0.231
	Control	6.651 ± 0.844^{c}	**Treatment*Replicate**	4	10.5	3.505	**0.008**

Terpenoids							
β-Pinene	Bumblebee	3.244 ± 0.208	Treatment	2	0.4	0.179	0.842
	Hoverfly	3.536 ± 0.204	Replicate	2	0.7	0.263	0.781
	Control	2.969 ± 0.111	**Treatment*Replicate**	4	5	10.216	**<0.001**
α-Farnesene	Bumblebee	695.463 ± 42.837	Treatment	2	0.9	0.283	0.767
	Hoverfly	663.227 ± 28.190	Replicate	2	1.5	0.498	0.641
	Control	711.422 ± 36.929	**Treatment*Replicate**	4	6.2	5.365	**<0.001**
Sulphur containing compounds							
1-Butene-4-isothiocyanate	Bumblebee	36.913 ± 3.074	Treatment	2	0.9	0.574	0.604
	Hoverfly	45.387 ± 8.056	Replicate	2	1.5	0.981	0.450
	Control	33.004 ± 2.952	Treatment*Replicate	4	3	1.309	0.267
Nitrogen-containing aromatics							
Benzyl nitrile	Bumblebee	95.450 ± 8.903	Treatment	2	52.6	1.656	0.299
	Hoverfly	70.713 ± 6.558	Replicate	2	15.1	0.474	0.653
	Control	35.013 ± 3.832	**Treatment*Replicate**	4	63.5	22.136	**<0.001**
2-Aminobenzaldehyde	Bumblebee	612.147 ± 66.092	Treatment	2	232.4	2.270	0.219
	Hoverfly	186.996 ± 24.659	Replicate	2	107.7	1.052	0.430
	Control	353.000 ± 37.575	**Treatment*Replicate**	4	204.8	16.308	**<0.001**
Indole	Bumblebee	160.244 ± 14.063	Treatment	2	83.3	5.550	0.070
	Hoverfly	71.712 ± 10.444	Replicate	2	11.8	0.783	0.516
	Control	74.270 ± 5.425	**Treatment*Replicate**	4	30	7.403	**<0.001**
Methylanthranilate	Bumblebee	470.553 ± 36.892[a]	**Treatment**	2	73.2	14.762	**0.014**
	Hoverfly	242.353 ± 36.275[b]	Replicate	2	16.9	3.410	0.137
	Control	144.879 ± 11.431[b]	Treatment*Replicate	4	9.9	1.645	0.163
Fatty-acid derivates							
(*Z*)-3-Hexenyl acetate	Bumblebee	49.430 ± 5.027	Treatment	2	1.4	0.097	0.910
	Hoverfly	45.484 ± 9.810	Replicate	2	3.9	0.271	0.775
	Control	48.095 ± 4.514	**Treatment*Replicate**	4	28.7	12.820	**<0.001**
Sum Scent							
Total amount of scent	Bumblebee	2838.088 ± 154.835	Treatment	2	7.8	3.850	0.117
	Hoverfly	1948.000 ± 90.421	Replicate	2	1.1	0.532	0.624
	Control	1890.010 ± 78.781	**Treatment*Replicate**	4	4	5.086	**0.001**
Color							
PC1 (500-650nm)	Bumblebee	-0.527 ± 0.124	Treatment	2	3.3	0.244	0.794
	Hoverfly	-0.631 ± 0.113	**Replicate**	2	225.8	16.708	**0.011**
	Control	-0.422 ± 0.101	**Treatment*Replicate**	4	27	12.547	**<0.001**
PC2 (290-400nm)	Bumblebee	0.211 ± 0.099	Treatment	2	4.6	0.542	0.619
	Hoverfly	0.106 ± 0.084	Replicate	2	30.7	3.639	0.126
	Control	-0.075 ± 0.093	**Treatment*Replicate**	4	16.9	5.594	**<0.001**
PC3 (410-490nm)	Bumblebee	0.055 ± 0.083	Treatment	2	4.5	0.612	0.586
	Hoverfly	-0.172 ± 0.085	Replicate	2	7.9	1.093	0.418
	Control	-0.215 ± 0.044	**Treatment*Replicate**	4	14.5	7.333	**<0.001**
PC4 (260-280nm)	Bumblebee	0.464 ± 0.070	Treatment	2	13.4	0.855	0.491
	Hoverfly	0.943 ± 0.058	Replicate	2	11.6	0.737	0.534
	Control	0.857 ± 0.068	**Treatment*Replicate**	4	31.4	23.975	**<0.001**
Seed production							
Seeds per fruit	Bumblebee	8.360 ± 0.982[a]	**Treatment**	2	2451.7	9.265	**0.032**
	Hoverfly	5.420 ± 0.508[b]	Replicate	2	245.7	0.930	0.466
	Control	16.456 ± 1.315[c]	**Treatment*Replicate**	4	529.4	4.612	**0.002**
Seeds per visited plant	Bumblebee	84.450 ± 13.545	Treatment	1	36155.6	1.465	0.350
	Hoverfly	41.375 ± 6.138	Replicate	2	10336.9	0.209	0.827
			Treatment*Replicate	2	4937.1	6.403	**0.003**

Chapter II

Do more efficient pollinators drive floral trait evolution in plants with generalized pollination?

Daniel DL Gervasi, Florian P Schiestl

Left: Bumblebee (*Bombus terrestris*) visit of *Brassica rapa* flowers, right: hoverfly (*Episyrphus balteatus*) visit to *B. rapa* flower.

Abstract

In nature, plants show to be visited and pollinated by a wide array of different pollinators. Stebbins' most effective pollinator principle states that the most effective pollinators impose the strongest selection on floral traits therefore determining floral adaptation (Stebbins 1970). In this experimental evolution study, we examine Stebbins' principle in the generalized plant *Brassica rapa*, which was exposed to a mixed pollinator environment comprised of bumblebees (*Bombus terrestris*) and hoverflies (*Episyrphus balteatus*). Pollinator-mediated selection and trait evolution was measured on floral traits such as flower morphology, color and scent. We found that the different pollinator species differed in few preferences but strongly in their efficiency. The most efficient pollinator, namely bumblebee, did not determine pollinator-mediated selection and floral trait evolution based on its preferences. Moreover, evolutionary changes in plant traits were found to occur but without clear detectable selection by the pollinators, suggesting that other factors may have played a more important role in trait evolution. This study found low support for Stebbins' most effective pollinator principle and suggests that low efficient pollinators may influence selection on floral traits more than indicated trough their low contribution to pollination.

Introduction

Angiosperms show a staggering diversity in floral color, scent and morphology. It has been suggested, that one of the major mechanisms behind this floral diversity is the adaptations of plants to different pollinators (Grant and Grant 1965; Stebbins 1970; Johnson 2006; Harder and Johnson 2009; Van der Niet et al. 2014). This diversity is possible as pollinator types differ in their preferences, foraging behavior, body morphology and the way they perceive sensory signals (Lunau et al. 2011; Newman et al. 2012; Schiestl and Johnson 2013). Additionally, these pollinators vary also in their geographical distribution creating a pollinator mosaic (Herrera et al. 2006; Johnson 2006). As a consequence, variations in pollinator environments can cause divergent pollinator-mediated selection, and with it the evolution of distinct floral phenotypes adapted to the local pollinator environments in allopatry or parapatry (Galen 1989; Johnson and Steiner 1997; Johnson 2006; Gomez et al. 2009; Johnson 2010; Sun et al.

2014; Gross et al. 2016). Moreover, in highly specialized pollination systems such adaptations to different pollinators have been indicated to even occur in sympatry (Xu et al. 2011; Schiestl 2012). Furthermore, comparative analyses of angiosperm lineages have shown that pollinators are one of the main drivers of plant diversification due to their essential role in plant mating (Dodd et al. 1999; Van der Niet and Johnson 2012). Additionally, the important role of pollinators in floral adaptation can also be seen in the convergent evolution of floral traits (termed 'pollination syndromes') found in different plant families (Fenster et al. 2004).

However, in nature plant-pollinator systems are often dominated by generalized interactions (Waser et al. 1996; Memmott 1999). This raises the question as to how floral diversification and adaptations to pollinators evolved when plants are often visited by a wide array of different pollinators with different preferences. Stebbins addressed this paradox and proposed that floral traits will be mainly shaped by the pollinator that is most efficient and abundant (Stebbins 1970). This implies that the strongest selection is mainly imposed by pollinators with the highest visitation frequency and the most efficient pollen transfer rate, which assumes a strong relationship between these two factors. As a condition for plants to evolve specialization, the pollinators must thus vary in their effectiveness (Schemske and Horvitz 1984). Since the proposal of Stebbins' "most effective pollinator principle" numerous studies have been conducted on the effectiveness of different pollinators and confirm that the most common pollinator is also the most efficient one as proposed by Stebbins (Motten et al. 1981; Fishbein and Venable 1996; Olsen 1997; Fenster and Dudash 2001; Sahli and Conner 2007; Zych et al. 2013). However, other studies also show that it is often the case that rare or uncommon pollinators are the most efficient vectors, contradicting the link between quantity and quality in pollinators (Schemske and Horvitz 1984; Ramsey 1988; Olsen 1997; Mayfield et al. 2001; Watts et al. 2012; Roque et al. 2016). The conclusion is thus that pollination efficiency and visitation frequency show a rather weak relationship. Moreover, it has been argued that instead of effectiveness, rather fitness trade-offs will determine if a plant evolves to specialize (Aigner 2001). This may lessen the importance of pollinator effectiveness on floral trait diversification. Nevertheless, it is still important to focus on pollinator effectiveness, as it remains crucial in the evolution of floral diversification (Fenster et al. 2004).

Of importance in Stebbins' most effective pollinator principle is that the most effective pollinator imposes the strongest selection (Stebbins 1970). However, it has

been hypothesized that the presence of one species may alter the selection imposed by another one (termed "non-additive selection"), therefore changing the net selection (Sahli and Conner 2011). Furthermore, temporal and spatial fluctuations in pollinator compositions have been described as a major force preventing consistent selection and therefore floral specialization towards specific pollinators (Gomez and Zamora 2006; Johnson 2006). But it could be shown that pollinators are able to impose consistent selective pressure in generalized plant systems and that diverse pollinators can be assigned to few functional groups, which share similar properties and may therefore impose similar selective pressures (Fenster et al. 2004; Gomez et al. 2015). Unfortunately, there is a lack of experimental evolution data concerning floral specialization or the evolutionary consequences on floral traits in a generalist plant with pollinators differing in preference, selection, and effectiveness.

Using an experimental evolution approach with the generalist plant *Brassica rapa* and a mixed pollinator environment, we address the issue of floral adaptation in a generalized plant system. In a previous experimental evolution study with *B. rapa*, we were able to show that plant traits evolved in response to the pollinator group they were exposed to (Chapter 1). The pollinators (bumblebees and hoverflies) differed in the selection they imposed and also in their pollination efficiency (Chapter 1). This previous experiment represented an allopatric scenario where the plants and pollinators were spatially separated. However in nature we often encounter generalist plants simultaneously visited by multiple different pollinators. The aim of this study was to observe the effects on plant trait evolution in the generalist *B. rapa* under a constant sympatric bumblebee and hoverfly environment. Specifically, we wanted to answer the following questions: 1) Do we find differences in floral trait preferences and pollination efficiency between the pollinators; 2) Do preferences of the most efficient pollinator transfer into phenotypic selection; and 3) Do we find floral trait evolution (specialization) corresponding to the preferences of the most efficient pollinator?

Materials and Methods

Plants

In this study, fast cycling *Brassica rapa* (Brassicaceae; Wisconsin Fast Plants®) was used as a model plant. These outcrossing plants have a short generation time about 35-40 days (Tomkins and Williams 1990). An initial population of 300 seeds (Wisconsin Fast Plants® Standard Seed) were obtained from Carolina Biological Supply (Burlington, NC, USA), and grown in a phytotron under standardized soil, light and watering conditions. Of these 300 plants, 150 pairs were created and in total 108 full sib seed families generated by artificial crossing. We used only seed families from crosses where both parents produced fruits. These 108 full sib seed families represented the population at the start of the experiment. Hoverflies (*Episyrphus balteatus,* Katz Biotech AG, Germany) and bumblebees (*Bombus terrestris,* Biocontrol, Andermatt, Switzerland) were used in the experiment as pollinators. Both are known to be pollinators of Brassicaceae plants (Jauker and Wolters 2008; Rader et al. 2009).

For the starting population, a single member of every seed family was sown out. The experiment therefore consisted of 108 plants (representing 108 seed families), which we subdivided into three replicates (A, B, and C), each containing 36 plants of one family each. Plants of all replicates were raised in the phytotron under standardized soil (Einheitserde® classic, Einheitserde Werkverband e.V., Germany), light (24 hours light) and watering conditions. For each plant, traits were measured every second generation for seven generations and in total four data points were taken (G1, G3, G5, G7), with the exception of floral scent. Scent collection was only obtained from generations 3 (G3), 5 (G5) and 7 (G7); floral scent data from generation 1 was lost due to technical problems. Replicate floral scent data for generation 1 was obtained after the end of the experiment by re-growing plants from the starting generation and collecting scent from one plant from each of the 108 seed families. From the first generation in total 108 plants (36 from each replicate) were thus sampled for floral scent and were identical to the data used in (Chapter 1).

Plant traits

All morphological, scent and color traits were measured as described in (Chapter 1) and briefly summarized here. Before pollination experiments, petal width, length, stigma height and flower diameter of three randomly chosen flowers per plant were measured with a digital caliper. Nectar of three randomly chosen flowers was collected with 1µl glass capillaries (Blaubrand, Wertheim, Germany) and the volume was measured. The

mean of each of these traits across the three flowers was used for the analysis. On the day of pollination experiments, the number of open flowers and plant height for each plant was measured just prior to pollination experiments.

Floral volatiles were collected before pollination from all plant inflorescences as soon as at least five flowers were open using non-destructive headspace sorption with a push-pull system. A detailed description of the collection method and analysis can be found in Chapter 1. Additionally, on the same day (but after pollination) the color reflectance spectra of three petals from different flowers for each plant were recorded using a fiberoptic spectrophotometer (AvaSpec-2048; Avantes, Apeldoorn, The Netherlands) and a xenon pulsed light source (AvaLight-XE; Avantes). A detailed description of the method can be found in Chapter 1. Across the entire experiment, plant traits of a total of 519 plants were collected and analyzed.

Self-compatibility

To test for self-compatibility after seven generations, we selfed three flowers per plant from each seed family of generation 7 of every replicate (in total 29 plants). The mean number of seeds per crossed flower for each individual plant was used as a measurement of self-compatibility. Self-compatibility data from the first generation is identical to Chapter 1.

Pollinator experiments

Bioassays with pollinators were performed in a flight cage (2.5m x 1.8m x 1.2m) in the greenhouse under standardized light conditions with bumblebees and hoverflies together. The bioassays were performed between 9am and 3pm from February 2013 till December 2014, using plants 23 days post sowing out. Bumblebees (*Bombus terrestris,* Biocontrol, Andermatt, Switzerland) were purchased as complete hives and held in the greenhouse while hoverflies (*Episyrphus balteatus,* Katz Biotech AG, Germany) were purchased as pupae and reared until eclosure, after which male and female flies were separated. The pollinators were fed with *B. rapa* plants, supplemental sugar water and pollen (Biorex, Ebnat-Kappel, Switzerland) until three days prior to experiments, after which only supplemental pollen and sugar water was used. Sixteen hours before the bioassays, supplemental sugar water and pollen were removed.

Plants in every replicate were randomly placed in a square of 6 x 6 plants with a distance of 20 cm from each other in the flight cage. Individual bumblebees (only workers) and hoverflies (males and females in equal quantities) were added simultaneously into the flight cage and allowed to visit a maximum of 3 different plants, after which the pollinators were removed from the cage; each insect was used only once. In total, 12-16 different plants per replicate received one or more visits by pollinators, at a maximum eight plants for each pollinator group. Pollinators were used until the needed number of visited plants was reached. Bumblebees that were inactive for 5 minutes and hoverflies inactive for 15 minutes were removed and replaced with fresh ones. Identity of visited plants, number of visits and number of visited flowers were recorded for each pollinator. Based on the ratio between number of open flowers and number of visited flowers, the percentage of visited flowers per plant and pollinator was calculated. After the bioassay, visited flowers were marked and plants were kept in an insect-proof cage for an additional 30 days until fruits were ripe for harvest. Seeds were counted and relative seed set was calculated for each plant as following: individual seed set/plot mean seed set. The average seed weight for each visited plant was also measured. Additionally, for each visited plant pollination efficiency was calculated as the number of seeds per fruit. For the next generation a total of 36 plants were grown from these seeds. The seed contribution of each visited plant into the next generation was calculated as: 36/(replicate sum of seeds/individual seed set) for each replicate. Furthermore, for each replicate in every generation the germination rate of all sown out seeds was quantified (for the last generation the germination rate was not quantified).

Statistics

Trait preferences:

To test if bumblebees or hoverflies showed preferences for certain plant traits, mean trait values were compared with a *t*-test between bumblebee visited ($N_{morphology\ and\ color}$ = 95, N_{scent} = 72) and not-visited ($N_{morphology\ and\ color}$ = 316, N_{scent} = 230) plants, respectively hoverfly visited ($N_{morphology\ and\ color}$ = 97, N_{scent} = 74) and not-visited plants ($N_{morphology\ and\ color}$ = 314, N_{scent} = 228). Prior to the analysis, scent data and nectar volume data were ln(1+x) transformed to approach normal distribution. All plant traits were then z-transformed to a mean of 0 and standard deviation of 1 for each replicate in every generation separately. Additionally, to reduce the high number of color variables, a

principal component analysis (PCA) with varimax rotation was performed on the standardized color variables. For subsequent analysis only principal components (PCs) with an eigenvalue above one were used. The PCA resulted in four such PCs explaining 97.261% of the total variance in flower color. *T*-tests were then performed for each standardized trait between bumblebee visited and non-visited plants, hoverfly visited and not-visited plants respectively over all replicates and generations for increased statistical power.

Additionally, trait preferences of bumblebees and hoverflies were also measured in an allopatric setting, with only one pollinator species present. For this, data from Chapter 1 of the bumblebee and hoverfly treatment was used (for material and methods used see Chapter 1). Preferences were calculated as above by *t*-test between mean trait values (prepared as above) of non-visited and bumblebee visited plants, respectively hoverfly visited plants (Bumblebee: $N_{visited} = 229$, $N_{non\text{-}visited} = 398$; Hoverfly: $N_{visited} = 235$, $N_{non\text{-}visited} = 235$).

Pollinator-mediated phenotypic selection:

In a similar approach to (Schiestl et al. 2011; Parachnowitsch et al. 2012) selection differentials (S) and selection gradients (β) were calculated with univariate (for S) and multivariate (for β) generalized linear models (glm) as the covariance between plant trait(s) and plant fitness (effectiveness, see below). The plant trait variables were divided into three trait-groups for the multivariate models and analyzed separately: a) morphology and nectar (flower number, plant height, stigma height, petal length and width, flower diameter, nectar per flower), b) color (wavelengths 260nm-650nm) and c) floral scent (14 scent compounds). Scent and nectar amounts were ln(1+x) transformed to approach normal distribution. Standardization of the data was performed as described above as well as variable reduction of color variables through PC-analysis. For the measurement of selection gradients, the data of all replicates and generations were combined to increase statistical power. Selection differentials were then calculated using univariate glms (poisson distribution with log link) for each trait over all replicates and generations. The selection gradients were calculated using multivariate glms (poisson distribution with log link) for each trait-group separately also over all replicates and generations. Additionally, disruptive/stabilizing selection gradients were measured using the multivariate model, with the squared term of each trait also added to the model. Effectiveness was used as a proxy for plant fitness (the dependent

variable) to avoid zero-inflated relative seed set values and because hoverfly and bumblebees differ in their efficiency. It was calculated as the product of number of visits and pollination efficiency (seeds per fruit), which was for this calculation divided in 5 categories (0 = 0 seeds per fruit, 1 = 0.1-10 seeds per fruit, 2 = 10.1 – 20 seeds per fruit, 3 = 20.1 – 30 seeds per fruit and 4 = 30.1 – 40 seeds per fruit). Effectiveness showed a significant correlation with relative seed set ($r_{411} = 0.723$, $p < 0.001$) and was therefore appropriate to use as a fitness proxy.

Evolutionary changes:

Evolutionary changes of traits over generations were measured as a linear regression with the trait as the dependent variable and "generation" as the independent (explanatory) variable. This was done for every replicate separately. Evolutionary changes over time of a trait were only considered to be supported if the changes were significant in a regression analysis in either all three replicates (consistent evolution) or two replicates (less consistent evolution), and followed the same direction in each case (regression coefficient either always positive or always negative). Additionally, traits needed to have undergone sufficient divergent adaptation after seven generations. This was determined using a univariate general linear model between the first and the seventh generation. For the general linear model (full factorial), trait was used as the dependent variable, generation as a fixed factor, and replicate as a random factor. Scent amounts and nectar volume were ln(1+x) transformed to approach normal distribution. For the general linear model with the color variables, a PCA was performed as described above, but variables were not standardized. The PCA was performed for all replicates and all generations together, resulting in four PCs explaining 96.298% of the total variance in floral color. For fecundity and pollination efficiency, only the values of visited plants were used and both values were ln(1+x) transformed. The condition for sufficient divergent selection were met when the factor "generation" or the interaction "generation*replicate" (or both) were significant. Evolutionary changes in traits that did not fulfill the above mentioned criteria were considered to be generated by drift.

Pollinator efficiency and fecundity:

For comparisons of pollination efficiency (number of seeds per fruit) and fecundity (seed set), only values of plants visited by pollinators were taken into account. Comparisons were performed over all generations and replicates combined. For

statistical analysis a univariate general linear model (with *post-hoc* LSD) was used. As dependent variable seeds per fruit or seed set was taken, pollinator (bumblebee, hoverfly or visited by both) as fixed factor, generation and replicate as random factor. Interactions among the factors were incorporated into the analysis to test for variability among the generations and replicates. Prior to analysis pollination efficiency and fecundity were ln(1+x) transformed to approach normal distribution. Additionally, comparisons between the percentage of open flowers visited by bumblebees, hoverflies and both pollinators were done by a one-way ANOVA (with *post-hoc* LSD).

Seed quality:

The average seed weight per visited plant was compared among the generations with all replicates combined by a one-way ANOVA with LSD *post-hoc* test. The seed germination rate was also compared among the generations with all replicates combined by a one-way ANOVA with LSD *post-hoc* test.

Self-compatibility:

Differences in self-compatibility (mean number of seeds per selfed flower) between the first and seventh generations (with replicates combined) were analyzed using a t-test.

Statistics were performed with IMB SPSS Statistics (Version 22.0.0, http://www-01.ibm.com/software/analytics/spss/products/statistics/).

Results

Preferences

Of all visited plants, 17% were visited by both pollinators, while 41% were chosen only by bumblebees and 42% only by hoverflies. Pollinators were found to visit plants preferably mainly due to morphological attributes (Table 1). Both pollinators preferred tall plants with many flowers (Table 1). Hoverflies additionally preferred also plants that produced more nectar per flower (Table 1). Concerning floral scent, only bumblebees showed preferences, specifically for plants with increased *p*-anisaldehyde and low 1-butene-4-isothiocyanate emissions (Table 1). Additionally, only bumblebees showed a preference for a color PC representing reflectance wavelengths from 290-400nm (Table 1).

In an allopatric setting (only one pollinator species present), bumblebees and hoverflies showed both similar strong preferences in morphological traits, while on floral scent mainly bumblebees showed preferences, and no preferences were found on floral color (Table S1). Both pollinators were found to prefer tall plants with many big flowers (Table S1). Bumblebees showed significant preferences for plants that emit phenylacetaldehyde, methyl benzoate, *p*-anisaldehyde, indole and methyl anthranilate in high amounts, while plants with low emissions of 1-butene-4-isothiocyanate were preferred (Table S1). Hoverflies showed only preferences for plant with low emissions of z-3-hexenylactetae (Table S1).

Table 1: Bumblebee and hoverfly preferences shown as mean trait values (± s.e.). Numbers in bold indicate significant differences between visited and not-visited plants for the corrsponding trait and pollinator (*t*-test, $p < 0.05$).

Trait	Pollinator	Visited	Not-visited
Morphology and Nectar			
Flower number	Bumblebee	**13.063 ± 0.522**	**10.867 ± 0.282**
	Hoverfly	**13.454 ± 0.617**	**10.732 ± 0.260**
Stigma heigth	Bumblebee	0.470 ± 0.101	0.467 ± 0.054
	Hoverfly	0.469 ± 0.095	0.467 ± 0.055
Petal length	Bumblebee	0.605 ± 0.007	0.593 ± 0.004
	Hoverfly	0.604 ± 0.006	0.594 ± 0.004
Petal width	Bumblebee	0.510 ± 0.006	0.502 ± 0.004
	Hoverfly	0.511 ± 0.006	0.501 ± 0.004
Flower diameter	Bumblebee	1.379 ± 0.014	1.349 ± 0.008
	Hoverfly	1.378 ± 0.015	1.349 ± 0.008
Plant heigth	Bumblebee	**26.381 ± 0.480**	**24.900 ± 0.314**
	Hoverfly	**26.192 ± 0.506**	**25.949 ± 0.312**
Nectar per flower	Bumblebee	114.096 ± 13.160	95.404 ± 5.073
	Hoverfly	**124.929 ± 13.249**	**91.939 ± 14.961**
Scent			
Aromatics			
Benzaldehyde	Bumblebee	300.033 ± 29.052	316.641 ± 17.731
	Hoverfly	326.769 ± 34.476	308.109 ± 16.708
Phenylacetaldehyde	Bumblebee	49.342 ± 14.699	37.411 ± 4.590
	Hoverfly	51.751 ± 16.451	36.524 ± 3.799
Methyl benzoate	Bumblebee	40.147 ± 5.285	45.954 ± 4.355
	Hoverfly	51.374 ± 8.820	42.361 ± 3.726
Phenylethyl alcohol	Bumblebee	3.154 ± 0.360	3.747 ± 0.297
	Hoverfly	3.343 ± 0.371	3.691 ± 0.297
Methyl salicylate	Bumblebee	25.639 ± 3.279	24.352 ± 1.546
	Hoverfly	22.889 ± 2.101	25.233 ± 1.741
p-Anisaldehyde	Bumblebee	**12.757 ± 2.631**	**8.633 ± 0.898**
	Hoverfly	8.101 ± 1.804	10.108 ± 1.085
Terpenoids			
β-Pinene	Bumblebee	2.624 ± 0.184	2.915 ± 0.110
	Hoverfly	2.622 ± 0.174	2.918 ± 0.112
α-Farnesene	Bumblebee	558.308 ± 42.282	590.702 ± 30.334
	Hoverfly	607.595 ± 56.627	574.989 ± 27.897
Sulfur-containing compounds			
1-Butene-4-isothiocyanate	Bumblebee	**16.879 ± 2.271**	**23.077 ± 2.372**
	Hoverfly	19.430 ± 2.618	22.304 ± 2.356
Nitrogen-containing aromatics			
Benzyl nitrile	Bumblebee	36.114 ± 4.918	30.588 ± 1.888
	Hoverfly	28.092 ± 3.454	33.143 ± 2.185
2-Aminobenzaldehyde	Bumblebee	382.126 ± 60.565	260.524 ± 21.976
	Hoverfly	309.750 ± 59.753	282.948 ± 22.271
Indole	Bumblebee	49.776 ± 7.102	36.744 ± 2.512
	Hoverfly	36.235 ± 5.252	41.025 ± 2.946
Methyl anthranilate	Bumblebee	115.542 ± 15.041	111.900 ± 10.304
	Hoverfly	100.472 ± 13.040	116.759 ± 10.600
Fatty-acid derivate			
(Z)-3-Hexenyl acetate	Bumblebee	26.228 ± 3.283	29.807 ± 1.988
	Hoverfly	23.204 ± 2.075	30.820 ± 2.142
Color			
PC1 (490-650nm)	Bumblebee	-0.025 ± 0.107	0.007 ± 0.056
	Hoverfly	0.116 ± 0.107	-0.036 ± 0.055
PC2 (290-400nm)	Bumblebee	**0.174 ± 0.093**	**-0.052 ± 0.057**
	Hoverfly	0.113 ± 0.099	-0.035 ± 0.057
PC3 (410-480nm)	Bumblebee	0.043 ± 0.110	-0.013 ± 0.055
	Hoverfly	0.013 ± 0.091	-0.004 ± 0.058
PC4 (260-280nm)	Bumblebee	-0.052 ± 0.104	0.016 ± 0.056
	Hoverfly	-0.091 ± 0.098	0.028 ± 0.057

Pollinator-mediated selection

Overall, directional pollinator-mediated selection was found to be weak (Table 2). Significant selection differentials were found for plant morphology as well as color and to a lesser extent for scent compounds. Pollinators were found to select for taller plants with more flowers, which were themselves larger and contained more nectar (Table 2). Within the scent compounds, *β*-pinene, (*Z*)-3-hexenyl acetate and 1-buten-4-isothiocyanate were found to be under significant negative selection by the pollinators (Table 2). Pollinators showed significant positive selection on the color PC comprising reflectance values from 290-400 nm.

In the multivariate analysis, selection gradients were found to be slightly weaker than the selection differentials (Table 2). Flower number was found to be under significant positive selection, while stigma height was under significant negative selection (Table 2). Within floral scent, significant negative selection on *β*-pinene and benzyl nitrile was found, but no other volatiles were under selection (Table 2). Additionally, significant positive selection on the color PC comprising reflectance values from 290-400 nm was found (Table 2). The pollinators showed stabilizing and disruptive selection on morphological and scent traits (Table 2). Petal width, methyl salicylate and *p*-anisaldehyde were found to be under significant stabilizing selection while 2-aminobenzaldehyde was under significant disruptive selection (Table 2).

Table 2: Selection differentials (S), gradients (β) and quadratic gradients (γ) with standard error (± s.e.) for all traits. Numbers in bold indicate signifcant selection based on a univariate (S) and multivariate (β,γ) generalized linear model ($p < 0.05$)

Trait	S ± s.e.	β ± s.e.	γ ± s.e.
Morphology and nectar			
Flower number	**0.376 ± 0.052**	**0.328 ± 0.059**	-0.079 ± 0.044
Stigma heigth	-0.069 ± 0.060	**-0.137 ± 0.069**	-0.074 ± 0.055
Petal lenght	**0.173 ± 0.061**	0.138 ± 0.078	-0.009 ± 0.062
Petal width	0.062 ± 0.060	-0.139 ± 0.078	**-0.153 ± 0.066**
Flower diameter	**0.198 ± 0.061**	0.165 ± 0.110	0.050 ± 0.051
Plant height	**0.239 ± 0.061**	0.101 ± 0.069	0.050 ± 0.048
Nectar per flower	**0.236 ± 0.069**	0.131 ± 0.072	0.081 ± 0.038
Scent			
Aromatics			
Benzaldehyde	0.100 ± 0.070	0.172 ± 0.089	-0.035 ± 0.056
Phenylacetaldehyde	-0.014 ± 0.071	0.072 ± 0.113	0.070 ± 0.059
Methylbenzoate	0.120 ± 0.069	0.128 ± 0.103	-0.024 ± 0.064
Phenylethyl alcohol	-0.100 ± 0.071	-0.081 ± 0.088	-0.092 ± 0.072
Methyl salicylate	0.042 ± 0.071	0.039 ± 0.086	**-0.308 ± 0.088**
p-Anisaldehyde	0.107 ± 0.070	0.076 ± 0.078	**-0.220 ± 0.084**
Terpenoids			
β-Pinene	**-0.206 ± 0.073**	**-0.315 ± 0.085**	-0.018 ± 0.062
α-Farnesene	0.086 ± 0.072	0.027 ± 0.077	-0.008 ± 0.056
Sulfur-containing compounds			
1-Butene-4-isothiocyanate	-0.137 ± 0.075	-0.111 ± 0.085	0.084 ± 0.051
Nitrogen-containing aromatics			
Benzyl nitrile	-0.043 ± 0.071	**-0.249 ± 0.123**	0.019 ± 0.081
2-Aminobenzaldehyde	0.102 ± 0.072	0.178 ± 0.131	**0.165 ± 0.083**
Indole	-0.007 ± 0.071	-0.031 ± 0.107	0.001 ± 0.071
Methyl anthranilate	0.100 ± 0.073	0.052 ± 0.116	0.094 ± 0.060
Fatty-acid derivates			
(Z)-3-Hexenyl acetate	**-0.154 ± 0.074**	-0.062 ± 0.085	-0.102 ± 0.066
Color			
PC1 (520-650 nm)	-0.074 ± 0.059	-0.075 ± 0.059	-0.016 ± 0.047
PC2 (290-400 nm)	**0.158 ± 0.062**	**0.158 ± 0.062**	-0.054 ± 0.059
PC3 (410-500 nm)	0.036 ± 0.058	0.032 ± 0.059	0.010 ± 0.036
PC4 (260-280 nm)	-0.082 ± 0.059	-0.075 ± 0.058	-0.073 ± 0.047

Trait evolution

During seven generations, several plant traits underwent evolutionary change (Table 3). Major morphological changes occurred in plant height and the number of open flowers, both decreasing strongly (Table S2). Comparison of the first and last generation revealed significant generation x replicate interactions for number of open flowers, plant height and the stigma height, showing differences between the first and seventh generation in these traits varied strongly among the replicates (Table S3).

Floral scent was also found to have changed significantly (Table 3). Major changes occurred within the aromatics and the nitrogen-containing aromatic compounds. Of the

aromatics, benzaldehyde and methyl salycilate were found to have increased, while phenylacetaldehyde had decreased (Table S2). Within the nitrogen-containing aromatics, 2-aminobenzaldehyde, indole, and methyl anthranilate were found to had decreased over time (Table S3). After seven generations methyl anthranilate was found to be emitted in much lower amounts then in the first generation (Table S3). Most other traits, with the exception of 1-butene-4-isothiocyanate, (Z)-3-hexenyl acetate and methyl benzoate, had a significant generation x replicate interaction, indicating that between the first and seventh generation differences in these traits varied strongly among the replicates (Table S3).

Flower color also showed major changes during the experiment. The PC-factor comprising reflectance values from 490-650nm was found to decrease strongly, while reflectance values from 260-280 nm increased and showed a significant generation x replicate interaction, showing that differences among the generations in these reflectance values varies strongly between the replicates (Table S2 and S3).

Table 3: List of plant traits that fullfilled the criteria for evolutionary changes. Significant linear regression in all three replicates are shown as +++/--- and in two replicates are shown as +/-. Significant differences between first and seventh generation based on general linear model (GLM) are show as "g" when factor "generation" was significant and # if interaction "generation x replicate" was significant.

Trait	Regression	GLM
Morphology and nectar		
Number of open flowers	-	#
Plant heigth	-	#
Scent		
Aromatics		
Benzaldehyde	+	#
Phenylacetaldehyde	-	#
Methyl salicylate	+	#
Nitrogen-containing aromatics		
2-Aminobenzaldehyde	-	#
Indole	-	#
Methylanthranilate	-	g
Color		
PC1 (490-650nm)	---	g
PC4 (260-280nm)	+	#

Pollination efficiency and fecundity

Overall, fecundity (as measured by seed production per visited plant) did not undergo evolutionary changes with ongoing generations (Table S2 and S3). The same was true for pollination efficiency, which also did not increase over time (Table S2 and S3).

Comparisons between the individual pollinator groups revealed that fecundity (seed set) was on average 4.7 times higher for bumblebee-pollinated plants than in hoverfly-pollinated ones (Fig 1A). Additionally, the interaction pollinator x generation was found to be significant, showing that the differences in fecundity between the pollinators varied among the generations ($F_{6,145}$ = 2.854, p = 0.012). Pollination efficiency (seeds per fruit) was found to be on average 1.9 times higher for bumblebees than for hoverflies (Fig 1B). Differences in pollination efficiency between the pollinators, varied also strongly between the generations as the interaction pollinator x generation was found to be significant ($F_{6,145}$ = 2.168, p = 0.049). Plants visited by both pollinators showed similar fecundity and pollination efficiency as plants visited by only bumblebees (Fig 1A and B). Additionally, bumblebees were found to visit on average 93 % of the open flowers of a plant, while hoverflies visited only 49 % (Fig 1C).

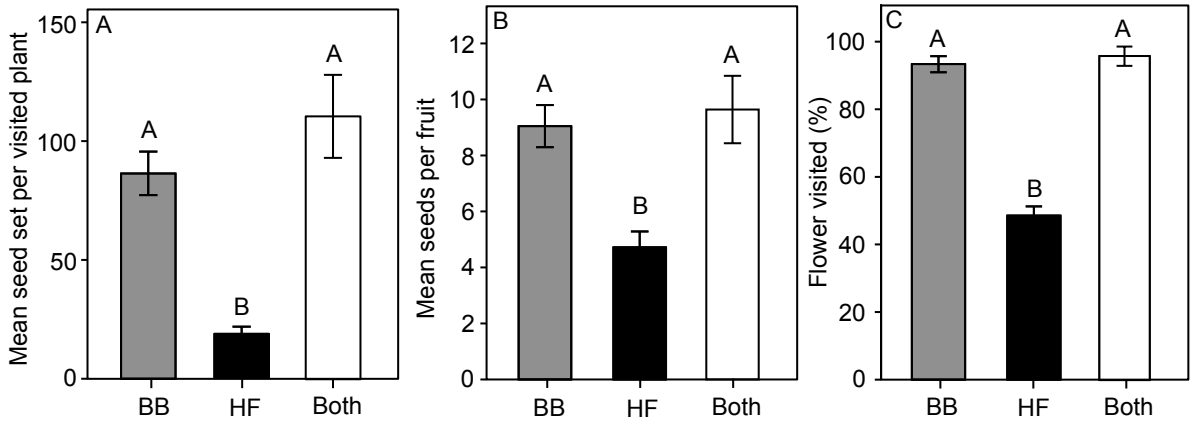

Fig 1: A) Mean fecundity (± s.e), B) mean pollination efficiency (± s.e.), and C) mean percentage of open flowers visited per plant (± s.e) of plants visited by bumblebees (BB), hoverflies (HF) and both pollinators (Both) over all generations and replicates. Different letters indicate significant differences among treatments (LSD *post-hoc* tests, p < 0.001).

Seed quality

The average seed weight differed significantly among the generations with a significant drop in the last generation (Fig S1A, $F_{3,159} = 3.252$, $p = 0.023$). The germination rate was found to no differ between the generations (Fig S1B, $F_{2,6} = 2.043$, $p = 0.211$).

Self-compatibility

Self-compatibility was found to increase significantly in seventh generation plants compared to the first generation plants (Fig S2, $t_{95} = 5.434$, $p < 0.001$).

Discussion

It has been suggested that adaptations of plants to different pollinators are one of the major mechanism for generating todays' floral diversity (Grant and Grant 1965; Stebbins 1970; Johnson 2006; Harder and Johnson 2009; Van der Niet et al. 2014). These distinct pollinators are assumed to select for different floral traits, which causes plant traits to diverge (Waser and Campbell 2004). Indeed, pollinators have been shown to differ in their morphology, behavior, and preferences, which can lead plants to adapt to the local pollinator environments (Galen et al. 1987; Johnson and Steiner 1997; Gomez et al. 2009). However as plants are often visited by a wide array of pollinators (Waser et al. 1996), Stebbins proposed that selection by the most effective pollinator should be the strongest force shaping floral traits (Stebbins 1970). In our study, we show, using an experimental evolution approach, that the most efficient pollinator did not dictate the selection regime on floral traits and that floral trait evolution did not follow the most efficient pollinator's preferences.

Do pollinators differ in preferences and efficiency?

It has been shown that pollinators differ in their morphology, food requirements, innate and learned preferences, and the way floral signals are perceived by them and therefore can exhibit preferences toward different floral signals (Galen et al. 1987; Lunau 2000; Lunau et al. 2011; Newman et al. 2012; Schiestl and Johnson 2013). In our study, overall both pollinators showed preferences for morphological traits while only bumblebees showed preferences for floral scent compounds. These similar preferences on

morphological traits by both pollinators and on floral scent by only bumblebees are in agreement to ones found in the allopatric pollinator setting. Bumblebees are known to use floral scent for discriminating plants and learn it (especially in combination with visual cues) to find rewarding plants (Galen and Newport 1987; Kunze and Gumbert 2001; Knauer and Schiestl 2015). Syrphids in general are assumed to use visual cues for finding food and having preferences for the color yellow (Sutherland et al. 1999; Shi et al. 2009), but it could be shown that they also can use olfactory cues (or both) for finding food (Laubertie et al. 2006; Primante and Dötterl 2010). However, as *B. rapa* has yellow flowers is it likely that hoverflies used this color as major cue and may have ignored floral scent, explaining why only bumblebees showed preferences for floral scent.

Bumblebees are known to be important and efficient pollinators for many plant species, often used commercially for greenhouse crop pollination (Chapter 1; (Winter et al. 2006). Hoverflies have also been described to be efficient pollinators in crops (Jauker and Wolters 2008). In this study bumblebees showed higher pollination efficiency and plants visited by them also had much higher seed set (fecundity). Plants visited by both pollinators showed a similar pollination efficiency and fecundity as only-bumblebee-visited plants, indicating that hoverflies did not contribute much to pollination efficiency and fecundity. This is in agreement to a previous study, where it was shown that bumblebees were significantly more efficient pollinators than hoverflies, and plants visited by bumblebees also had a higher fecundity (Chapter 1). Following Stebbins' most effective pollinator principle, we would expect that floral evolution would be driven by the bumblebees' preferences, as their higher efficiency are expected to transfer into stronger selection. Moreover, the impact of bumblebees on floral trait evolution should be intensified as they additionally visited more flowers per plant and therefore bumblebee-visited plants produced significantly more seeds.

Does higher efficiency transfer into stronger selection?

Based on Stebbins' most effective pollinator principle, in our experiment the *B. rapa* plants should be found to adapt towards the preferences of the more effective of our two pollinators, as it should impose stronger selection (Stebbins 1970). In our study we found that the preferences on morphological traits that both pollinators showed, did transfer into selection for these traits. This was further confirmed trough the strong overlap of pollinator preferences and the selection on the corresponding traits in an

allopatric pollinator setting (see Chapter 1, Table S2). Especially, selection for increased flower production has been shown to be quite common in selection studies (Harder and Johnson 2009). However, preferences for floral scent in the bumblebees did not transfer into stronger selection for these traits. In the case of *p*-anisaldehyde we found it to be actually under stabilizing selection. This was surprising as bumblebees have been shown to impose strong selection with corresponding evolutionary changes on this floral scent compound, while hoverflies showed none (Chapter 1). It is possible that the hoverflies, albeit being a less efficient pollinator, may have altered the selection imposed by bumblebees. In such a scenario, where the presence of a pollinator may alter the selection of another pollinator, selection is thought to be non-additive (Sahli and Conner 2011). For 1-butene-4-isothiocyanate, the bumblebee preferences did not transfer into negative selection, although there was a negative trend. For the color PC comprising the reflectance values from 290-400nm, the preferences of the bumblebees did transfer into selection. These findings indicate that higher efficiency does not necessarily transfer into stronger selection and in our case that a low efficient pollinator can influence selection. But as bumblebees and hoverflies in this study only showed preferences for few floral traits, it is difficult to devise a general statement on the effect that more efficient pollinators have on selection.

Pollinator-mediated selection and floral trait evolution

In general we found that several plant traits have undergone evolutionary changes over time but these changes did not follow exactly pollinator-mediated selection (e.g. flower number, plant height, methyl anthranilate). This indicates that other, stronger processes were impacting trait evolution beside selection. It could be, that these traits may have undergone evolutionary changes due to pleiotropy with other unmeasured traits under selection. It has been shown in *B. rapa* that pleiotropic effects exist among floral traits such as scent and morphology (Zu et al. 2015). Alternatively, it has been shown that inbreeding depression can have strong negative impacts on plant vigor (Charlesworth and Charlesworth 1987; Dudash et al. 1997; Andersson 2012). But the comparison of germination rates, which was relatively high over the generations, indicates that inbreeding depression might have not played an essential role in the floral evolution. However, the comparison of average seed weights shows a significant drop in

the last generation, which could indicate that the plants performance was strongly reduced and therefore may partially explain the evolutionary changes.

Conclusion

In our study we could show that higher pollination-efficiency does not transfer into stronger selection and that evolutionary changes in floral traits may occur without corresponding pollinator-mediated selection. In further experimental evolution studies, special emphasis should be therefore set allowing pollinators to impose strong enough selection to better see the effects of different efficient pollinators. This may further help us to shed more light into the process of floral adaptions in generalized pollination systems.

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Supplementary Material

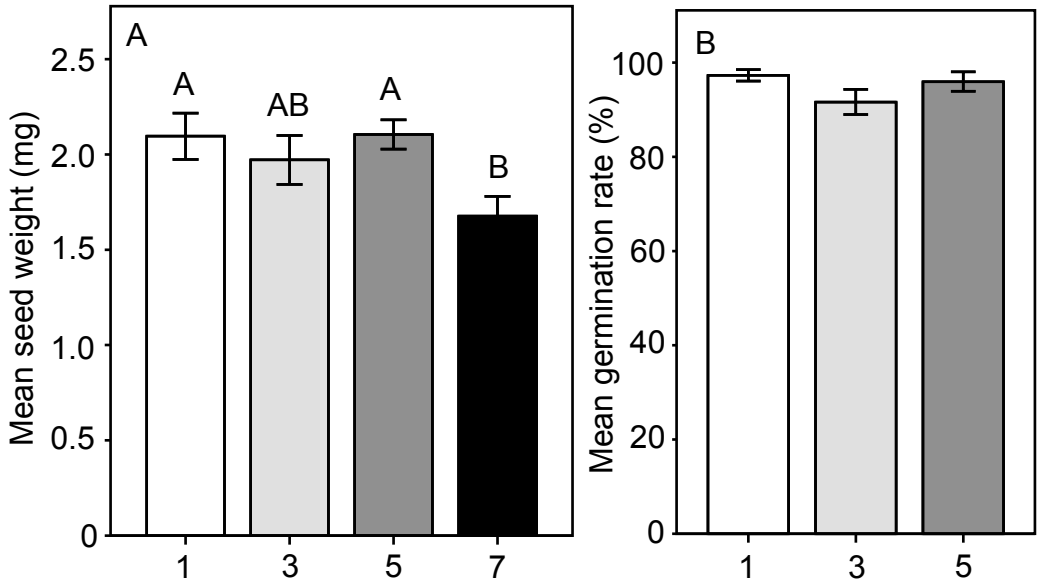

Fig S1: (A) Mean seed weight (± s.e.) of visited plants, and (B) mean replicate germination rate (± s.e.) for multiple generations (x-axis) over all replicates combined. Different letters above the bar indicate significant differences (LSD *post-hoc*, $p < 0.05$)

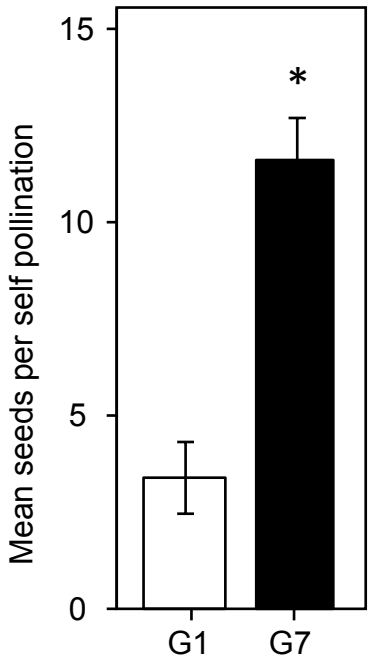

Fig S2: Self-compatibility of plants as the mean number of seeds per self pollinated flower (± s.e) in the first generation (G1) and plants form the seventh generation (G7). Asterisks above the bar represent significant differences (t-test, $p < 0.001$).

Table S1: Bumblebee and hoverfly preferences in an allopatric setting as mean trait values (± s.e.). Numbers in bold indicate significant differences between visited and not-visited plants for the corrsponding trait and pollinator (*t*-test, $p < 0.05$).

Trait	Pollinator	Visited	Not-visited
Morphology and Nectar			
Flower number	Bumblebee	**12.921 ± 0.367**	**9.878 ± 0.234**
	Hoverfly	**10.934 ± 0.312**	**9.208 ± 0.249**
Stigma heigth	Bumblebee	0.432 ± 0.079	0.424 ± 0.060
	Hoverfly	0.415 ± 0.069	0.417 ± 0.058
Petal length	Bumblebee	**0.593 ± 0.004**	**0.576 ± 0.066**
	Hoverfly	**0.587 ± 0.004**	**0.579 ± 0.004**
Petal width	Bumblebee	**0.522 ± 0.005**	**0.496 ± 0.074**
	Hoverfly	0.471 ± 0.005	0.471 ± 0.004
Flower diameter	Bumblebee	**1.340 ± 0.010**	**1.311 ± 0.008**
	Hoverfly	**1.330 ± 0.009**	**1.298 ± 0.008**
Nectar per flower	Bumblebee	105.385 ± 5.844	95.090 ± 4.935
	Hoverfly	129.020 ± 8.200	118.249 ± 6.596
Plant heigth	Bumblebee	**27.633 ± 0.367**	**24.273 ± 0.265**
	Hoverfly	**22.738 ± 0.308**	**21.572 ± 0.278**
Scent			
Aromatics			
Benzaldehyde	Bumblebee	224.268 ± 15.168	237.595 ± 12.881
	Hoverfly	246.288 ± 17.516	260.613 ± 13.124
Phenylacetaldehyde	Bumblebee	**88.530 ± 12.747**	**75.168 ± 10.914**
	Hoverfly	82.671 ± 18.799	63.011 ± 7.396
Methyl benzoate	Bumblebee	**74.168 ± 10.412**	**63.870 ± 7.451**
	Hoverfly	67.341 ± 5.125	79.835 ± 6.611
Phenylethyl alcohol	Bumblebee	4.470 ± 0.512	4.046 ± 0.285
	Hoverfly	3.997 ± 0.373	4.258 ± 0.329
Methyl salicylate	Bumblebee	30.357 ± 2.377	27.254 ± 2.262
	Hoverfly	23.913 ± 1.770	24.516 ± 1.358
p-Anisaldehyde	Bumblebee	**11.860 ± 1.321**	**9.697 ± 0.827**
	Hoverfly	3.533 ± 0.431	4.345 ± 0.462
Terpenoids			
β-Pinene	Bumblebee	3.048 ± 0.172	2.906 ± 0.126
	Hoverfly	3.333 ± 0.203	3.594 ± 0.166
α-Farnesene	Bumblebee	532.081 ± 33.268	559.907 ± 26.798
	Hoverfly	578.312 ± 30.443	578.513 ± 24.345
Sulfur-containing compounds			
1-Butene-4-isothiocyanate	Bumblebee	**18.976 ± 2.048**	**23.740 ± 1.829**
	Hoverfly	28.828 ± 5.198	38.861 ± 5.880
Nitrogen-containing aromatics			
Benzyl nitrile	Bumblebee	68.027 ± 6.016	60.743 ± 3.865
	Hoverfly	75.627 ± 9.816	67.834 ± 4.282
2-Aminobenzaldehyde	Bumblebee	539.314 ± 46.915	459.713 ± 30.857
	Hoverfly	255.287 ± 26.526	257.356 ± 24.085
Indole	Bumblebee	**120.747 ± 10.524**	**97.905 ± 5.649**
	Hoverfly	66.445 ± 6.218	73.658 ± 6.076
Methyl anthranilate	Bumblebee	**275.182 ± 22.423**	**241.718 ± 18.150**
	Hoverfly	151.403 ± 19.107	241.882 ± 26.794
Fatty-acid derivate			
(Z)-3-Hexenyl acetate	Bumblebee	30.485 ± 2.618	32.622 ± 2.454
	Hoverfly	**30.050 ± 2.756**	**44.401 ± 5.930**
Color			
PC1 (510-650nm)	Bumblebee	0.014 ± 0.078	0.97 ± 0.059
	Hoverfly	0.044 ± 0.073	0.001 ± 0.052
PC2 (290-400nm)	Bumblebee	0.194 ± 0.067	0.087 ± 0.062
	Hoverfly	0.070 ± 0.067	0.052 ± 0.054
PC3 (410-500nm)	Bumblebee	0.088 ± 0.071	0.175 ± 0.067
	Hoverfly	-0.049 ± 0.062	-0.090 ± 0.057
PC4 (260-280nm)	Bumblebee	-0.107 ± 0.064	-0.142 ± 0.048
	Hoverfly	0.051 ± 0.067	0.123 ± 0.060

Table S2: Univariate linear regression analysis with plant trait as dependent variable and generation as independent variable for each replicate. Replicates with significant regression in a plant trait are shown in bold.

Trait	Replicate	Coefficient ± s.e.	*t*	Sig.
Morphology and nectar				
Flower number	**A**	**-0.434 ± 0.176**	-2.464	**0.015**
	B	-0.221 ± 0.183	-1.207	0.23
	C	**-1.045 ± 0.192**	-5.439	**<0.001**
Stigma heigth	**A**	**0.149 ± 0.034**	4.328	**<0.001**
	B	-0.030 ± 0.031	-.946	.346
	C	0.059 ± 0.039	1.524	.130
Petal length	A	-0.001 ± 0.003	-.593	.554
	B	**-0.005 ± 0.002**	-2.511	**.013**
	C	0.001 ± 0.003	.318	.751
Petal width	A	0.004 ± 0.002	1.578	.117
	B	-0.001 ± 0.002	-.640	.523
	C	0.004 ± 0.003	1.642	.103
Flower diameter	A	-0.005 ± 0.006	-.904	.368
	B	-0.006 ± 0.005	-1.178	.241
	C	0.007 ± **0.006**	1.280	.203
Plant heigth	A	-0.291 ± 0.181	-1.604	.111
	B	**-0.869 ± 0.175**	-4.968	**<0.001**
	C	**-0.896 ± 0.220**	-4.075	**<0.001**
Nectar per flower	**A**	**0.111 ± 0.040**	2.801	**.006**
	B	**-0.122 ± 0.053**	-2.297	**.023**
	C	0.079 ± 0.045	1.775	.078
Scent				
Aromatics				
Benzaldehyde	**A**	**0.143 ± 0.025**	5.7566	**<0.001**
	B	**0.095 ± 0.025**	3.7071	**<0.001**
	C	-0.004 ± 0.032	-0.1218	.903
Phenylacetaldehyde	A	-0.052 ± 0.043	-1.2078	.229
	B	**-0.146 ± 0.038**	-3.8058	**<0.001**
	C	**-0.168 ± 0.042**	-4.0333	**<0.001**
Methyl benzoate	**A**	**-0.077 ± 0.031**	-2.4738	**.015**
	B	0.013 ± 0.029	0.4494	.654
	C	0.006 ± 0.040	0.1511	.880
Phenylethyl alcohol	**A**	**0.142 ± 0.025**	5.6387	**<0.001**
	B	0.043 ± 0.025	1.7600	.081
	C	0.008 ± 0.037	-0.2065	.837
Methyl salicylate	**A**	**0.089 ± 0.027**	3.3436	**.001**
	B	**0.065 ± 0.029**	2.2741	**.025**
	C	0.002 ± 0.044	0.0511	.959
p-Anisaldehyde	**A**	**0.119 ± 0.044**	2.6876	**.008**
	B	**-0.096 ± 0.033**	-2.9631	**.004**
	C	0.020 ± 0.046	0.4305	.668

Terpenoids				
β-Pinene	**A**	**0.077 ± 0.014**	5.7222	**<0.001**
	B	0.024 ± 0.013	1.7851	.076
	C	0.003 ± 0.016	0.1748	.862
α-Farnesene	A	-0.013 ± 0.028	-0.4615	.645
	B	**0.067 ± 0.023**	2.8918	**.004**
	C	**-0.220 ± 0.036**	-6.0749	**<0.001**
Sulfur-containing compounds				
1-Butene-4-isothiocyanate	A	-0.019 ± 0.032	-0.5790	.564
	B	0.021 ± 0.030	0.6999	.485
	C	0.005 ± 0.034	0.1441	.886
Nitrogen-containing aromatics				
Benzyl nitrile	A	-0.002 ± 0.029	-0.0670	.947
	B	**-0.141 ± 0.027**	-5.2092	**<0.001**
	C	-0.064 ± 0.039	-1.6438	.103
2-Aminobenzaldehyde	A	0.070 ± 0.051	1.3865	.168
	B	**-0.230 ± 0.052**	-4.4228	**<0.001**
	C	**-0.292 ± 0.071**	-4.1321	**<0.001**
Indole	A	-0.070 ± 0.046	-1.5177	.131
	B	**-0.219 ± 0.046**	-4.7140	**<0.001**
	C	**-0.178 ± 0.045**	-3.9810	**<0.001**
Methyl anthranilate	**A**	**-0.104 ± 0.048**	-2.1691	**.032**
	B	**-0.180 ± 0.044**	-4.0722	**<0.001**
	C	-0.086 ± 0.050	-1.7161	.088
Fatty-acid derivate				
(*Z*)-3-Hexenyl acetate	A	0.022 ± 0.026	0.8500	.397
	B	0.051 ± 0.026	1.9597	.052
	C	0.036 ± 0.029	1.2457	.215
Color				
PC1 (490-650nm)	**A**	**-0.080 ± 0.034**	-2.372	**0.019**
	B	**-0.165 ± 0.038**	-4.296	**<0.001**
	C	**-0.099 ± 0.035**	-2.836	**0.005**
PC2 (290-400nm)	A	0.018 ± 0.036	.509	0.611
	B	**0.082 ± 0.033**	2.521	**0.013**
	C	-0.018 ± 0.042	-.433	0.666
PC3 (410-480nm)	A	0.015 ± 0.026	.566	0.572
	B	-0.021 ± 0.028	-.761	0.448
	C	**0.183 ± 0.047**	3.875	**<0.001**
PC4 (260-280nm)	A	0.009 ± 0.038	.236	0.813
	B	**0.183 ± 0.030**	6.172	**<0.001**
	C	**0.152 ± 0.037**	4.141	**<0.001**
Seed production				
Seeds per fruit	A	-0.054 ± 0.048	-1.127	.265
	B	0.038 ± 0.047	.805	.424
	C	0.013 ± 0.036	.368	.714
Seeds per visited plant	A	-0.121 ± 0.083	-1.465	.149
	B	0.132 ± 0.077	1.716	.092
	C	-0.020 ± 0.064	-.305	.761

Table S3: Comparison of plant traits in the first and seventh generation with a univariate general linear model. Floral volatiles are shown in ρg*l^{-1}. Factors or interactions that have a significant effect are shown in bold.

Trait	Generation	Mean ± s.e.	Factors	df	Type III SOS	*F*	Sig.
Morphology and nectar							
Flower number	1	13.259 ± 0.466	Generation	1	592.64	5.443	0.145
	7	9.934 ± 0.463	Replicate	2	138.75	0.637	0.611
			Generation*Replicate	2	217.77	4.987	**0.008**
Stigma heigth (cm)	1	0.464 ± 0.085	Generation	1	2.42	0.753	0.477
	7	0.485 ± 0.086	Replicate	2	6.47	1.006	0.499
			Generation*Replicate	2	6.43	4.403	**0.013**
Petal length (cm)	1	0.620 ± 0.005	Generation	1	0.03	9.175	0.094
	7	0.595 ± 0.005	Replicate	2	0.03	4.541	0.180
			Generation*Replicate	2	0.01	1.164	0.314
Petal width (cm)	1	0.502 ± 0.005	Generation	1	0	0	0.996
	7	0.501 ± 0.006	Replicate	2	0.04	2.84	0.260
			Generation*Replicate	2	0.01	2.163	0.118
Flower diameter (cm)	1	1.392 ± 0.015	Generation	1	0.06	1.683	0.324
	7	1.359 ± 0.010	Replicate	2	0.02	0.357	0.737
			Generation*Replicate	2	0.07	1.891	0.153
Plant heigth (cm)	1	29.467 ± 0.438	Generation	1	1059.5	9.503	0.091
	7	24.994 ± 0.492	Replicate	2	297.4	1.334	0.429
			Generation*Replicate	2	223	5.28	**0.006**
Nectar per flower (nl)	1	70.594 ± 4.837	Generation	1	0.34	0.038	0.863
	7	92.190 ± 11.671	Replicate	2	3.54	0.195	0.837
			Generation*Replicate	2	18.14	13.238	**<0.001**
Scent							
Aromatics							
Benzaldehyde	1	389.745 ± 19.964	Generation	1	10.17	2.57	0.250
	7	580.200 ± 25.386	Replicate	2	2.92	0.368	0.731
			Generation*Replicate	2	7.92	17.941	**<0.001**
Phenylacetaldehyde	1	79.877 ± 14.385	Generation	1	35.73	7.352	0.113
	7	27.297 ± 4.556	Replicate	2	3.98	0.41	0.709
			Generation*Replicate	2	9.72	4.465	**0.013**
Methyl benzoate	1	87.377 ± 10.105	Generation	1	1.76	1.153	0.395
	7	70.669 ± 7.700	Replicate	2	10.1	3.306	0.232
			Generation*Replicate	2	3.05	2.96	0.054
Phenylethyl alcohol	1	5.565 ± 0.464	Generation	1	10.01	2.14	0.281
	7	7.691 ± 0.444	Replicate	2	16.4	1.754	0.363
			Generation*Replicate	2	9.35	26.791	**<0.001**
Methyl salicylate	1	35.323 ± 2.980	Generation	1	2.09	0.638	0.508
	7	39.173 ± 2.994	Replicate	2	0.95	0.145	0.873
			Generation*Replicate	2	6.55	5.555	0.004
p-Anisaldehyde	1	7.300 ± 0.909	Generation	1	0.21	0.044	0.853
	7	13.090 ± 2.202	Replicate	2	12.88	1.321	0.431
			Generation*Replicate	2	9.75	4.279	**0.015**

Terpenoids							
β-Pinene	1	2.752 ± 0.153	Generation	1	3.15	3.446	0.205
	7	3.715 ± 0.157	Replicate	2	0.92	0.505	0.665
			Generation*Replicate	2	1.83	7.405	**0.001**
α-Farnesene	1	895.912 ± 54.542	Generation	1	3.76	0.259	0.661
	7	759.070 ± 51.117	Replicate	2	11.46	0.394	0.717
			Generation*Replicate	2	29.07	31.817	**<0.001**
Sulfur-containing compounds							
1-Butene-4-isothiocyanate	1	30.651 ± 3.505	**Generation**	1	0.48	24.461	**0.038**
	7	33.468 ± 4.180	**Replicate**	2	3.04	78.306	**0.013**
			Generation*Replicate	2	0.04	0.037	0.963
Nitrogen-containing aromatics							
Benzyl nitrile	1	63.093 ± 5.437	Generation	1	12.46	2.778	0.237
	7	39.784 ± 4.125	Replicate	2	15.2	1.693	0.371
			Generation*Replicate	2	8.97	6.523	**0.002**
2-Aminobenzaldehyde	1	421.455 ± 38.492	Generation	1	38.03	1.251	0.38
	7	350.572 ± 50.017	Replicate	2	46.83	0.77	0.565
			Generation*Replicate	2	60.81	10.263	**<0.001**
Indole	1	92.823 ± 8.436	Generation	1	36.18	6.833	0.120
	7	50.635 ± 5.870	Replicate	2	13.89	1.311	0.433
			Generation*Replicate	2	10.59	4.069	**0.018**
Methylanthranilate	1	201.878 ± 20.583	**Generation**	1	11.25	29.078	**0.033**
	7	144.424 ± 16.834	Replicate	2	4.53	5.859	0.146
			Generation*Replicate	2	0.77	0.339	0.713
Fatty-acid derivates							
(Z)-3-Hexenyl acetate	1	41.135 ± 4.680	Generation	1	3.11	10.009	0.087
	7	44.862 ± 3.321	**Replicate**	2	17.5	28.144	**0.034**
			Generation*Replicate	2	0.62	0.737	0.480
Color							
PC1 (490-650nm)	1	0.225 ± 0.086	**Generation**	1	28.84	80.487	**0.012**
	7	-0.512 ± 0.081	**Replicate**	2	18.75	26.162	**0.037**
			Generation*Replicate	2	0.72	0.536	0.586
PC2(290-400nm)	1	-0.100 ± 0.097	Generation	1	5.87	5.287	0.148
	7	0.230 ± 0.084	Replicate	2	7.34	3.303	0.232
			Generation*Replicate	2	2.22	1.303	0.274
PC3 (410-480nm)	1	0.002 ± 0.062	Generation	1	1.33	0.143	0.741
	7	0.155 ± 0.099	Replicate	2	14.33	0.772	0.564
			Generation*Replicate	2	18.55	16.049	**<0.001**
PC4 (260-280nm)	1	-0.033 ± 0.083	Generation	1	2.54	0.354	0.612
	7	0.187 ± 0.076	Replicate	2	27.86	1.945	0.340
			Generation*Replicate	2	14.33	14.628	**<0.001**
Seed production							
Seeds per fruit	1	7.550 ± 0.944	Generation	1	0.28	0.409	0.588
	7	9.018 ± 1.277	Replicate	2	2	1.459	0.407
			Generation*Replicate	2	1.371	0.801	0.453
Seeds per visited plant	1	65.548 ± 12.529	Generation	1	2.263	0.38	0.601
	7	77.263 ± 15.218	Replicate	2	5.037	0.422	0.703
			Generation*Replicate	2	11.924	2.02	0.140

CHAPTER III

Mechanisms of reproductive isolation in a species pair of sexually deceptive orchids

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Left: *Ophrys aymoninii* and, right: *Ophrys insectifera*

Contributions in this Chapter:

Daniel Gervasi did perform and analyze the pollen tracking experiments in the field and measured fruiting success as well as embryo development of the intra-and interspecific crosses. Daniel Gervasi collected floral scent of the orchids in the field and analyzed it. Daniel Gervasi collected root samples for mycorrhizal association of both orchid species in the field and pollinia for ploidy analyses. Additionally, Daniel Gervasi performed behavioral assays with scent manipulated plants in the field and measured negative density-dependent selection. Daniel Gervasi conducted the statistical analyses and wrote the manuscript.
Florian Schiestl performed intra-and interspecific crosses between the two orchids. Florian Schiestl conducted the GC-EAD experiments and also performed behavioral assays with scent manipulated orchids in the field.
Marc-André Selosse identified and quantified the mycorrhizal associations of the two orchid species.
During the Masterthesis, the pollen tracking experiments, intra-and interspecific crosses and ploidy analysis were performed. During the PhD, further pollen tracking experiments were performed. Furthermore, floral scent and root samples for mycorrhiza associations of both orchid species in the field were collected. Additionally, behavioral essays with scent manipulated and negative density-dependent measurements were also performed in the field during the Ph.D. All analyses and the writing of the manuscript were done during the Ph.D.

Abstract

The evolution of isolation barriers is an important component of the process of speciation and plays a central part in the origin of biodiversity. Commonly, reproductive barriers are separated into pre- and postzygotic mechanisms that can evolve differently through time and in strength. In this study we measured the strength of these mechanisms in two closely related sexually deceptive orchids of the European *Ophrys insectifera* group, namely *Ophrys insectifera* and *Ophrys aymoninii*, in sympatric and allopatric populations. More specifically, we observed pollen flow, performed artificial inter- and intraspecific crosses, analyzed scent bouquets and mycorrhizal partners between the two species. Our results show that floral isolation trough attraction of specific pollinators acts as the major barrier between the two species while later acting barriers were found to be absent. Specifically, we found for the first time, that both species share the majority of mycorrhizal partners in sympatry. Key differences between the species were found in floral scent, mainly in two alkenes and ester, which we have shown in behavioral assays to be likely the key components in the attraction of male solitary bees. Our results show that both *Ophrys* species are reproductively isolated from each other in sympatry trough the attraction of different pollinators based on different odor bouquets and also may represent a promising candidate for sympatric speciation.

Introduction

In the light of the biological species concept, proposed by Ernst Mayr in 1942, an increasing number of studies have focused in past decades on the evolution of reproductive isolating barriers (Coyne and Orr 1989; Ramsey et al. 2003; Coyne and Orr 2004; Scopece et al. 2008). These barriers are essential for the reduction of gene flow and maintenance of reproductive isolation between species occurring in sympatry. Depending on the timing of their onset, reproductive isolation barriers are classified as either prezygotic (e.g. behavioral, mechanical or gametic isolation) or postzygotic (e.g. hybrid inviability or ecological inviability) (Coyne and Orr 2004). Often the maintenance of distinct species is caused by a combination of both types of barrier, but which barrier

has the greater impact is highly variable between taxa and strongly influenced by the properties of the reproductive biology (Coyne and Orr 1998; Rieseberg and Willis 2007). One of the major aspects in speciation research is to evaluate the relative importance of these different barriers, which can evolve at different rates and with different strengths (Coyne and Orr 1998; Ramsey et al. 2003; Coyne and Orr 2004; Lowry et al. 2008). An important objective for quantifying the strengths and importance of different barriers in speciation is the identification and measurement of all reproductive barriers between closely related sympatric species (Coyne and Orr 2004). Measurement of reproductive barriers in species that are distantly related may offer no information if or which barriers played a key role in speciation.

In plants, it has been hypothesized that the evolution of prezygotic barriers (e.g. floral isolation) often come first compared to postzygotic barriers and therefore play a critical part at the initial stages of reproductive isolation and maintaining species unity compared to postzygotic ones (Grant 1994; Kirkpatrick and Ravigne 2002; Coyne and Orr 2004; Moyle et al. 2004; Rieseberg and Willis 2007; Lowry et al. 2008; Widmer et al. 2009). These reproductive barriers at the initial stage of reproductive isolation are thought to involve genes of large effect, as formulated in the genic view of speciation (Wu 2001; Coyne and Orr 2004; Widmer et al. 2009). Indeed, genetic studies have shown that prezygotic barriers, most notably through floral isolation, can have a simple genetic basis strengthening the importance of prezygotic isolation mechanisms (Bradshaw and Schemske 2003; Hoballah et al. 2007; Schlüter et al. 2011; Xu et al. 2012a; Sedeek et al. 2014). Persuasive examples of the importance of prezygotic barriers are known from plant adaptations to different pollinator, where gene flow is reduced between plants as a consequence of attracting different pollen vectors (Ramsey et al. 2003; Kay 2006; Waelti et al. 2008; Widmer et al. 2009; Xu et al. 2011; Sedeek et al. 2014; Sun et al. 2015). These adaptations to different pollinators have been suggested to be driven by spatially and temporally distributions of pollinators (Johnson 2006) or by low fecundity as plants compete for pollinator access (Waser and Campbell 2004). As a consequence this competition for similar pollinators can result in negative density dependent selection, where dense populations suffer from low fecundity. In deceptive plants, where pollinators can learn to avoid the plants and fecundity is low, it is suggested to be a common situation where negative density dependent selection may favor the switching of pollinators even in sympatry (Tremblay et al. 2005; Xu et al. 2011, Gross and Schiestl unpublished data). Therefore, plant systems with high pollinator

specificity, especially deceptive systems where floral isolation is often a key barrier, have been found to provide good study systems for the evolution of prezygotic isolation barriers and ecological speciation (Peakall and Whitehead 2014; Sedeek et al. 2014).

In orchids, more specifically sexually deceptive orchids, prezygotic barriers in form of floral isolation are thought to play an important role in reproductive isolation (Scopece et al. 2007; Cozzolino and Scopece 2008; Schiestl and Schluter 2009; Ayasse et al. 2011; Xu et al. 2011; Peakall and Whitehead 2014; Sedeek et al. 2014). The pollination mechanism of sexually deceptive orchids, which are thought to encompass 11 genera with nearly 400 species worldwide known, has evolved multiple times in different lineages and represents an intriguing adaptation to pollinators (Cozzolino and Widmer 2005; Gaskett 2011). In sexually deceptive orchids the flowers attract and deceive male pollinators into pseudocopulations by mimicking mating signals, such as morphology and sex pheromones, that facilitate pollination (Schiestl et al. 1999; Jersakova et al. 2006; Schiestl and Schluter 2009; Peakall et al. 2010; Peakall and Whitehead 2014). In this form of floral mimicry, floral odor often plays a key role in attracting highly specific pollinators and maintaining species integrity trough floral isolation (Schiestl et al. 1999; Mant et al. 2005b; Xu et al. 2011; Sedeek et al. 2014). In the European orchid genus *Ophrys*, one of the best-studied system in the sexually deceptive orchids, these scent compounds consist of a blend of cuticular hydrocarbons (alkanes, alkenes), which also can have a simple genetic basis (Schlüter and Schiestl 2008; Xu et al. 2012a; Xu et al. 2012b). However, reproductive isolation has never been studied in a proper sister-species pair in the *Ophrys* genus, which may give better insights into the importance of the barriers in the process of speciation.

While the emphasis in sexually deceptive orchids has been strongly on pollinator adaptation as a driver for prezygotic barriers, nearly nothing is known about the effects of their mychorrhizal partner on reproductive isolation. Orchids strongly depend on fungi, in a way that opens the door to post-zygotic barriers. Their small endosperm-less seeds only germinate upon colonization by a soil fungus (Dearnaley et al. 2016), that provide nutrients to them supporting germination until they eventually become autotrophic. Orchids often evolved specific dependence on groups of fungi (e.g. belonging to Tulasnellaceae and Seredipitaceae), collectively called rhizoctonias (Dearnaley et al. 2013). It has been hypothesized that associations to specific mycorrhizal fungi may act as an extrinsic post-zygotic barrier by preventing the germination of hybrid seeds trough the lack of a fungal partner (Scopece et al. 2008;

Jacquemyn et al. 2011). Changes in mycorrhizal fungi have also been claimed to be potential drivers of orchid speciation (Otero and Flanagan 2006; Waterman and Bidartondo 2008; Bateman et al. 2014), and there is evidence, although limited, that the sharing of similar fungi can facilitate hybridization (Schatz et al. 2010).

Within the sexually deceptive orchids, the *Ophrys insectifera* group offers an excellent system for investigating reproductive barriers in a phylogenetic background. The monophyletic *O. insectifera* group consists of three species, namely *Ophrys insectifera*, *Ophrys subinsectifera* and *Ophrys aymoninii* (Devey et al. 2008; Breitkopf et al. 2015). *O. subinsectifera* and *O. aymoninii* are narrowly restricted allopatric endemics occurring in the Spanish Pyrenees and the southern Massif Central in France, respectively (Delforge 2005). Both endemics regularly occur in sympatry with the more widespread *O. insectifera*. In this study we quantified the strengths of the individual reproductive barriers between the putative sister-species *O. insectifera* and *O. aymoninii* in allopatric and sympatric populations in southern France. We measured reproductive isolation at three levels, namely pre-pollination prezygotic (floral isolation), post-pollination prezygotic (fruiting success) and postzygotic (embryo development and mycorrhizal fungi) in our study. For floral isolation, we focused on attraction of specific pollinators (behavioral isolation), because mechanical isolation is usually weak or absent between *Ophrys* with the same positioning of pollinia (which is the case in the here studied species). Additionally, the importance of floral scent in the maintenance of specific pollinator attraction was tested by behavioral assays with scent-manipulated plants. The aim of this study was to answer following specific questions: 1) Is floral isolation through attraction of specific pollinators the main reproductive barrier between *O. insectifera* and *O. aymoninii* and/or are mycorrhizal fungi a potential post-zygotic barrier between the two species? 2) Does floral scent differ between the two species and which scent compounds play a key role in attracting the specific pollinator?

Material and Methods

Study species and site

Ophrys insectifera (Fig. S1A), a European terrestrial orchid, has a wide distribution and is pollinated by males of two digger wasp species Fig. (S1B)(*Argagorytes mystaceus* and *A.*

campestris) (Kullenberg 1951; Delforge 2005). Its putative sister-species, *Ophrys aymoninii* (Fig. S1C) is a narrow endemic found in the southern Massif Central in France and pollinated by males of the solitary bee *Andrena combinata* (Fig. S1D)(Borg-Karlson et al. 1993). Our study has been performed in the Parc Naturel Régional des Grands-Causses in Aveyron, France during May/June 2010-2013 where the two sister species were flowering simultaneously. In total, seven populations were studied in these four years (Table S1). For a better visualization of the sympatric occurrence we collected GPS points of randomly selected plants of both species in the mixed populations (Fig S2).

Prezygotic isolation (floral isolation)

To measure the strength of pollinator-mediated floral isolation, an experimental approach with a plot design to quantify pollen flow between the endemic *O. aymoninii* and the local *O. insectifera* species was conducted. The experimental setup, which was located at the same localities as the naturally occurring plants, was as following. Each experimental site contained between 5-11 plots (size depending on the availability of plant material). The plots were set up along transects through the orchids' and pollinators' habitats. Every plot contained four *Ophrys* plants with stained pollinia in 15 ml falcon tubes filled with water (two from each species). The pollinia of each species was stained with distinct staining dyes, specifically 2 % (w/v) Trypan red (72210-25G, Sigma-Aldrich) and 1 % (w/v) brilliant green (B6756-100G, Sigma-Aldrich), as described in Xu et al. (2011). The plants in one plot were positioned in a square of 0.2 m distance to the next plant. The distance between each plot was 20 m. After 5 days the plants were checked and pollinia removal as well as deposition of massulae on stigmata were noted. Floral isolation (RI_{floral}) was calculated as 1 - (total number of interspecific pollination events / total number of intraspecific pollination events) (Scopece et al. 2007). The value can vary between 0 (no floral isolation) and 1 (total floral isolation). Living plants of both species were collected at the locations Avey2 – Avey8 (see coordinates in Table S2). For the plot experiment only plants with intact open unpollinated flowers were used. In 2010, two experimental transects were performed at the location Avey3 and one in Avey2 (in total 3 experimental transects). In 2011, one experimental transect each was performed at the locations Avey2 and Avey3 (in total 2 experimental transects). This was due to an extremely low number of flowering plants. In 2012, one experimental transect each was performed at the locations Avey2, Avey3,

Avey4 and Avey6 (in total 4 experimental transects).

Post-pollination prezygotic isolation

To measure the post-pollination prezygotic barrier, intra-/interspecific hand crosses were performed between the two species. Ten *O. insectifera* and seven *O. aymoninii* were used to conduct hand pollination experiments. Intraspecific and interspecific crosses were performed with each of the two species (no plant was self-pollinated). Post-pollination prezygotic isolation (RI_{PPI}) was quantified by counting number of fruits on inter- and intraspecific crosses. RI_{PPI} was calculated as 1 - (mean number of fruits in interspecific crosses / mean number of fruits in intraspecific crosses). In the cases where interspecific crosses performed better than the intraspecific crosses (resulting in a negative value for RI_{PPI}), the reproductive isolation value was set to zero Scopece et al. (2007). Finally, the fruits were collected when they were ripe and dried in silica-gel (Fluka). Seeds of these fruits were used for quantification of postzygotic isolation by counting the number of developed embryos.

Postzygotic isolation

Embryo development

For measurement of this component of postzygotic isolation a random sample of 300 seeds from each fruit was examined under the binocular microscope (Olympus SZH-ILLD) at 64 x magnification. Seeds with a well-developed embryo and those without or with weakly developed embryos were counted. Well-developed embryos were visible as black grains, which were coated by the transparent embryo sack. Weakly developed embryos also contained an embryo but they were transparent like the embryo sack and also smaller than well-developed ones. Postzygotic isolation due to absence of a developed embryo (RI_{embryo}) was calculated as 1 - (mean number of developed embryos in interspecific crosses / mean number of developed embryos in intraspecific crosses) similar to Scopece et al. (2007). In cases where interspecific crosses performed better than the intraspecific crosses, the reproductive isolation value was set to zero Scopece et al. (2007).

Molecular barcoding of mycorrhizal fungi

We collected root samples from 24 *O. insectifera* and 26 *O. aymoninii* individuals from five populations (Avey2-6, on average 8 plants per population) during the field season in May 2012. Roots of the orchids were carefully excavated and a ~1 cm long fragment of the root was removed. For each individual, roots were thoroughly washed and on average ten thin root sections (<0.2 µm in thickness) displaying mycorrhizal infection under a light microscope were harvested. DNA of the 396 resulting samples was extracted as in Schatz et al. (2010). Barcoding with the fungal ribosomal intergenic transcribed spacer (ITS) was performed using primers ITS1F and ITS4 (universal for fungi), ITS1 and ITS4Tul (specific for most tulasnelloids), as well as ITS1 and ITSTul2 (specific for some tulasnelloids, 5'-TTCTTTTCCTCCGCTGAWTA-3'), and thereafter sequenced as in Schatz et al. (2010). Operational taxonomic units (OTUs) were delineated at the 97% similarity threshold, taxonomically affiliated using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). To ascertain the phylogenetic position of Sebacinales OTUs, one longer sequence per each OTU was obtained with primers ITS3seb and TW13 as in Selosse et al. (2009). One representative sequence per OTU was deposited in GenBank (GB accession numbers KF871201-19)

Flower odor sampling and chemical analysis

Scent was collected from unpollinated and intact open flowers by cutting the labella and extracting it in a 4 ml glass vial (Supelco) filled with 0.5 mL dichloromethane (HPLC grade, Fluka) for one minute while gently shaking. Afterwards the labellum was removed and the samples stored at -28°C until analysis in a gas chromatograph. In total, scent extracts of 38 *O. aymoninii* and 48 *O. insectifera* plants were taken during 2012 and 2013 from 5 populations. For quantitative analysis of floral scent a gas chromatograph (Agilent 6890N; Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector (FID) was used. One µl of each scent sample together with one µl of octadecane (10 ng μl^{-1}) as internal standard was injected splitless at 50° C (1 min), followed by a programmed increase of the oven temperature to 300°C at a rate of 4°C min^{-1}. The GC was equipped with an Agilent 19091J-431 column (0.25 mm diameter, 0.25 µm film thickness, 15 m length); hydrogen was used as carrier gas with a flow rate of 2.0 ml min^{-1}. As heptacosane (C_{27}) and palmitic acid nonyl ester ($C_{24}H_{48}O_2$) were found to overlap in

their retention time and chromatogram, an additional step was needed to quantify their relative amount. We injected one µl of each scent sample together with one µl of octadecane (10 ng μl^{-1}) as internal standard, splitless at 50° C (1 min), followed by a programmed increase of the oven temperature to 230°C at a rate of 10°C min^{-1}, in a gas chromatograph (Agilent 6890N; Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector (FID). The GC was equipped with an Agilent J&W 123-7032 DB-Wax (0.32 mm diameter, 0.25 µm film thickness, 30 m length) column; hydrogen was used as carrier gas with a flow rate of 2.0 ml min^{-1}. Additionally, standards of heptacosane (C_{27}) and palmitic acid nonyl ester ($C_{24}H_{48}O_2$) were run for identification of the two compounds. Based on the peak areas, the ratios of heptacosane and palmitic acid nonyl ester were calculated for each sample and used to calculate the relative amount of each compound in every sample of the first GC-FID analysis. For identification of compounds some scent samples were run on an Agilent GC with mass selective detection (Agilent 5975C; Agilent Technologies, Santa Clara, CA, USA). As above one µl sample and one µl octadecane (1 ng μl^{-1}) as internal standard were injected into the GC-MS. To identify the compounds their mass spectra were compared in a NIST library with spectra from known reference compounds. For confirmation of the identified GC compounds the spectrum and retention time were compared with following standards for alkanes: tricosane (C_{23}), tetracosane (C_{24}), pentacosane (C_{25}), hexacosane (C_{26}), heptacosane (C_{27}), nonacosane (C_{29}); alkenes: (*Z*)-9-pentacosene [(*Z*)-9-C_{25}], (*Z*)-9-heptacosane [(*Z*)-9-C_{27}], (*Z*)-9-nonacosene [(*Z*)-9-C_{29}]; and esters: palmitic acid octyl ester ($C_{24}H_{48}O_2$), palmitic acid nonyl ester ($C_{24}H_{48}O_2$). Additionally, four unknown compounds and docosenamide were added into the analysis. In total 16 scent compounds were used for the analysis. For the analysis, the relative amount of each odor compound was calculated as the proportion of the total amount of all 16 scent compounds.

GC-EAD

Gas chromatographic analysis with electroantennographic detection (GC-EAD; Schiestl and Marion-Poll 2002) of floral extracts was performed using a gas chromatograph (Agilent 6890 N, Agilent Technologies, Palo Alto, CA, USA) equipped with a heated outlet for electroantennographic recordings (Effluent Conditioning Assembly , Syntech, Hilversum, the Netherlands). Antennal responses of *Andrena combinata* males were

measured via EAD. For EAD recordings, the tip of the excised antenna was abscised and the antenna was mounted between two glass capillaries filled with Ringer solution mounted on a micro-manipulator (Micro Manipulator MP-12, Syntech, Hilversum, the Netherlands). The electrode at the base of the antenna was grounded via an Ag/AgCl wire and the electrode at the distal end of the antenna was connected via a signal interface box (Syntech, Hilversum, the Netherlands) to a personal computer. Up to 5 µl of *O. aymoninii* flower extract were injected splitless at 50˚C (1 min) into the GC followed by heating to 300˚C with a rate of 10˚C min^{-1}. The GC was equipped with an HP-5 column (0.32 mm diameter, 0.25 µm film thickness, 30 m length) and a flame ionization detector (FID). Hydrogen was used as carrier gas. A GC effluent splitter (Agilent G2855 Deans Switching System, Agilent Technologies, Palo Alto, CA, USA) was used to direct 50 % of the eluate, which was admixed to a purified and humidified air stream, over the excised antenna. EAD signals and FID responses were simultaneously recorded using Syntech software. Compounds releasing EAD responses were identified by comparison of retention times of samples with those of synthetic standard compounds.

Behavioral assays

To test if addition of scent compounds on *O. insectifera* flowers could attract male *Andrena combinata* bees the following behavioral assay was used. Non-manipulated *O. insectifera* and *O. aymoninii* plants acted as negative (*O. insectifera* with solvent only) respectively positive control for the assays while on each flower of the manipulated *O. insectifera* plants 10 µl of a scent mixture in hexane (25 ng/µl, (*Z*)9-C25, 27 ng/µl, (*Z*)9-C27, 7 ng/µl palmitic acid nonyl ester and 5 ng/µl palmitic acid octyl ester) was added with a syringe. These four specific compounds were chosen, as they were found to be electrophysiological-active compounds in *O. aymoninii* and differed significantly between the two species. By supplementing these four compounds to *O. insectifera* flowers, the scent-manipulated flowers emitted these four compounds in similar amounts as *O. aymoninii* while the other scent compounds were not altered. Each experimental set up consisted of two plants from every treatment with equal number of open flowers. The plants were then placed in 15 ml falcon tubes filled with water along the patrol ways of the male *Andrena combinata* bees (brushes, pine trees) randomly in a distance of 0.2 m from each other. The number of approaches (male bees stop patrolling and fly towards the flower without landing) as well as landings were recorded. These

experiments were performed between 11am and 3pm at the population Avey2 (on five days) and Avey6 (on one day) during May and June 2013. A reciprocal experiment with the digger wasp pollinator of *O. insectifera* could not be done, as those pollinators were never observed in the field by us.

Ploidy level

In 2010 two pollinia were taken from one flower per plant of *O. insectifera* (N=12) and *O. aymoninii* (N=11) in France at the Avey 3 population. In total 23 samples were collected. Pollinia samples were prepared and ploidy levels were measured identical as described in Xu et al. (2011).

Density dependent selection and fecundity

To measure if sexually deceptive plants are under negative density-dependent selection, a survey of totally 157 *O. aymoninii* and 143 *O. insectifera* from all seven populations in 2013 was performed. Randomly chosen flowering plants were marked at the beginning of the season as well as the number of conspecific and heterospecific *Ophrys* plants within a 2m radius were counted. A month later the number of flowers and developed fruits of the marked plants were noted. Relative female reproductive success was calculated as individual fruit set divided by the populations mean fruit set. Additionally, the number of fruits per open flower for each marked plant was used as measurement of plants fecundity.

Statistical Analysis

Differences in fruiting success and embryo development between intra-and interspecific crosses were analyzed by two-sample *t*-test for each species. Differences in relative amount of individual floral scent compounds between the two species were analyzed through Mann-Whitney-U tests. In the behavioral assays the differences in approaches and landings among *O. insectifera*, *O. aymoninii* and scent manipulated *O. insectifera* plants were analyzed trough X^2-tests with Bonferroni correction for multiple comparisons ($\alpha = 0.017$).

Differences in the number of found mycorrhiza fungi between *O. insectifera* and *O. aymonii* were analyzed by generalized linear model with binomial distribution. Mycorrhiza abundance was used as dependent variable and species as explanatory variable. Due to absence or extremely low abundance of mycorrhizal fungi no statistics could be performed for T3 and S2.

Density dependent selection was measured for each species by linear regression between relative female reproductive success (fRS) and the number of conspecific plants within 2 m. For calculation of density dependent selection conspecific plants for each population were z-transformed (mean = 1, s.d. = 1). This was done to eliminate the effect of different population densities. Differences in fecundity were analyzed by Mann-Whitney-U tests between the two species.

All statistical analysis has been performed in SPSS 20.0 (IBM SPSS Statistics).

Results

Pre-pollination prezygotic isolation

Floral isolation (RI_{floral})

The flowering time of *O. aymoninii* and *O. insectifera* was found to overlap strongly (Daniel Gervasi field observation). Floral isolation, measured as the ratio between intra- and interspecific pollination events, was estimated by tracking stained pollinia of 980 flowers between *O. aymoninii* and *O. insectifera* plants. In the three years 29 flowers were pollinated with stained pollinia. Among these flowers, no interspecific pollen transfers was observed but only intraspecific transfers. More specifically, eight *O. insectifera* flowers and 21 *O. aymoninii* flowers received conspecific stained pollinia (Fig 1A). Thus, both species had an estimated RI_{floral} value of 1 indicating complete or at least very strong reproductive isolation between *O. aymoninii* and *O. insectifera*.

Post-pollination prezygotic isolation

Fruiting success (RI_{PPI})

Post-pollination, prezygotic isolation was measured as the fruiting success ratio from 26 hand-crosses. The six intra – and six interspecific crosses in *O. aymoninii* revealed no differences in the fruiting success (Fig 2B,), resulting in a RI_{PPI} value of zero, indicating an absence of a reproductive barrier at this stage. In *O. insectifera*, the six interspecific crosses had a higher fruiting success than the eight intraspecific (t_{12}=0.812, p = 0.433) resulting in a negative RI_{PPI} value (RI_{PPI}= -0.143) subsequently set to zero. Thus no reproductive barrier was found at this stage (Fig 2B).

Postzygotic isolation

Embryo development (RI_{embryo})

Postzygotic isolation based on embryo viability was estimated as the ratio of well-developed embryos from hand-crosses. In both species, interspecific crosses showed a tendency to higher yield of seeds with well-developed embryos than the intraspecific crosses, albeit not significant (Fig 2C; *O. aym*, t_{10} = 0.015, p = 0.988; *O. ins*, t_8 = 0.934, p = 0.377). For both species a neg. RI_{embryo} value was calculated (*O. ins* = -0.111, *O. aym* = -0.006) and therefore regarded them as zero indicating no reproductive isolation through reduced or absent embryo development.

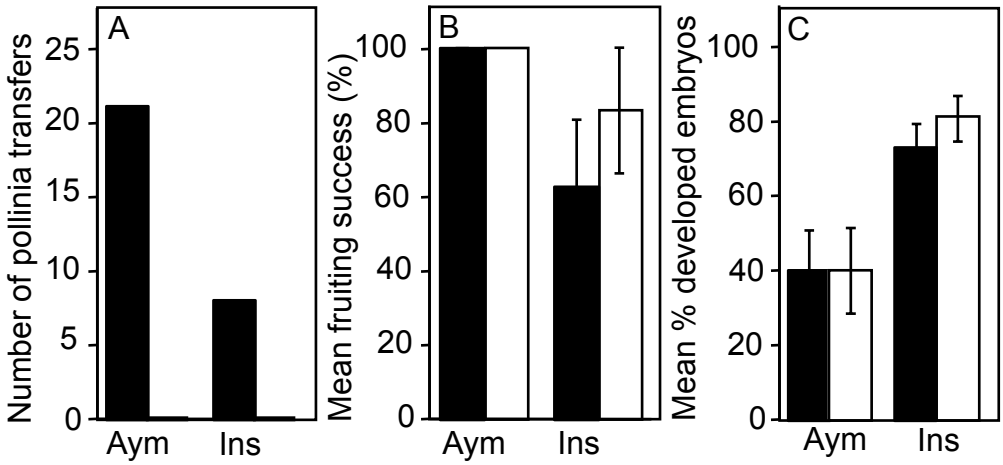

Fig. 1: Effectiveness of different reproductive barriers in *O. aymoninii* (Aym) and *O. insectifera* (Ins) trough (A) floral isolation, (B) mean prezygotic-postpollination isolation (± s.e.), and (C) mean postzygotic isolation (± s.e.). Bars in black stand for intraspecific pollinations/ crosses and white bars for interspecific pollinations/crosses. For B) and C) no differences were found between inter- and intraspecies crosses based on Student's t-test ($p > 0.05$).

Mycorrhizal fungi

Barcoding identified the *Tulasnellaceae* operational taxonomic unit (OTU) T1 (GB accession number KF871201) in 23 (out of 24) and 21 (out of 26) individuals of *O. insectifera* and *O. aymoninii*, respectively (Fig 3, Table S1). Other rhizoctonias included two Tulasnellaceae OTUs (T2 and 3; KF871202-3) and two Serendipitaceae OTUs (S1 and 2; KF871204-5; Table S1). All rhizoctonias OTUs were found on both host orchids, with exception of T3 (on one individual of *O. insectifera* individuals only) and S2 (on two *O. insectifera* individuals only; Fig 3). Barcoding also revealed OTUs of endophytic fungi (KF871206-14) or mycorrhizal on forest trees (ectomycorrhizal fungi, such as *Tricholoma*, *Rhizopogon* and *Russula*; KF871215-19; Table S1), unlikely to be truly orchid mycorrhizal (Dearnaley et al. 2013). GLM anlaysis revealed no difference in frequency of individuals with T1 and was found to be equally abundant on both hosts (df=1, Wald $X^2 = 0.011$; $p = 0.917$). This was also true for T2 (df=1, Wald $X^2 = 0.003$; $p = 0.954$), endophytic fungi (df=1, Wald $X^2 = 2.971$; $p = 0.085$) and ectomycorrhizal fungi

(df=1, Wald X^2 = 0.024; p = 0.877). Significant differences were only found for the Serendipitaceae S1 (Fig 3; df=1, Wald X^2= 6.392; p = 0.011). Based on this large overlap, differences of mycorrhizal partners are unlikely to favor reproductive isolation.

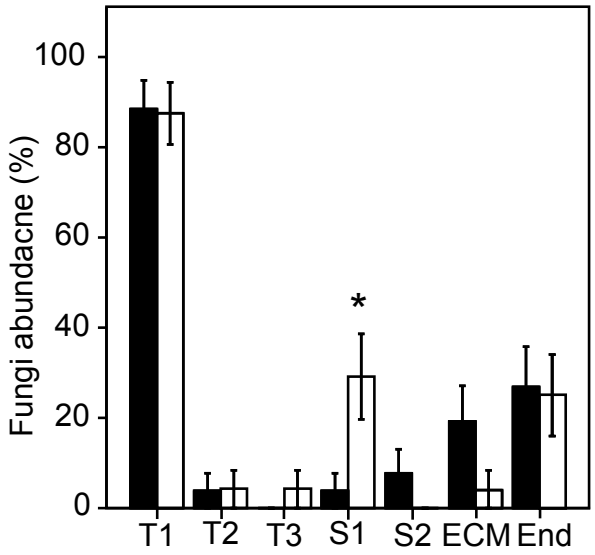

Fig. 2: Mean relative abundance (± s.e.) of specific fungi in *O. aymoninii* (black, N = 26) and *O. insectifera* (white, N = 24) plants. T1-3 = *Tulasnella*, S1-2 = Sebacinales, ECM = ectomycorrhizal fungi, End = endophytic fungi. Asterisk above the bar indicates significant differences in relative fungi abundance between the two species (GLM, p < 0.05).

Ploidy level analysis

Both species have been found to have the same, with one exception, ratios of relative fluorescence intensity between the internal standard and the pollinia (Fig S3). Thus, no overall difference in ploidy level was detected between the two species.

Scent composition and GC-EAD

Chemical analysis of the 16 most abundant floral scent compounds, of which 12 have been chemically identified, revealed strong differences between the two orchids species (Table 1). Out of these 16 compounds, 13 were found to differ significantly between the two species (Table 1). However, the most striking differences were found within the relative amounts of esters and alkenes. Palmitic acid octyl ester as well as palmitic acid nonyl ester was found in much higher amount in *O. aymoninii* (~ 5 times) while nearly absent in *O. insectifera* (Table 1). Within the alkenes (Z)-9-pentacosene and (Z)-9-heptacosene were also found to be present in much higher amounts in *O. aymoninii* than *O. insectifera* (Table 1). Additionally, these four compounds together with tricosane were found to be EAD active in *Andrena combinata* male bees while none of the other

compound elicited reproducible responses (Fig S4). No GC-EAD experiments were performed with the pollinators of *O. insectifera* as none of them could be obtained in the field.

Table 1: Mean relative scent amounts (± s.e.) of 16 scent compounds in *O. aymoninii* (N = 38) and *O. insectifera* (N = 46). Scent compounds in bold are electrophysiological active scent compounds based on GC-EAD with male *Andrena combinata* bees and *O. aymoninii* scent extracts. Different superscripts indicate significant differences in the rel. amount between the species (Mann-Whitney-U Test, $p < 0.05$).

Compounds	***O. aymoninii***	***O. insectifera***
Unknown 1	2.282±0.172[a]	3.644±0.273[b]
Tricosane	19.998±0.657[a]	19.929±0.820[a]
Tetracosane	2.925±0.172[a]	2.655±0.079[b]
(*Z*)-9-Pentacosene	7.415±0.561[a]	1.076±0.067[b]
Pentacosane	12.104±0.300[a]	13.683±0.369[b]
Palmitic acid octyl ester	0.675±0.063[a]	0.066±0.028[b]
Hexacosane	0.674±0.033[a]	0.994±0.042[b]
Unknown 2	1.183±0.046[a]	1.160±0.041[a]
(*Z*)-9-Heptacosene	19.448±0.693[a]	14.967±0.478[b]
Palmitic acid nonyl ester	2.217±0.152[a]	0.429±0.091[b]
Unknown 3	2.635±0.155[a]	3.776±0.184[b]
Heptacosane	4.524±0.121[a]	7.119±0.279[b]
Docosenamid	5.074±1.168[a]	5.173±1.105[a]
Unknown 4	9.340±0.595[a]	12.795±0.742[b]
(*Z*)-9-Nonacosene	8.397±0.466[a]	10.738±0.429[b]
Nonacosane	1.112±0.052[a]	1.797±0.118[b]

Behavioral assays

The behavioral assays revealed that the four GC-EAD active scent compounds (palmitic acid octyl ester, palmitic acid nonyl ester, (*Z*)-9-pentacosene and (*Z*)-9-heptacosene) in *O. aymoninii* were crucial in the attraction of its pollinator *Andrena combinata* and hence likely mediate floral isolation. Scent manipulated *O. insectifera* flowers had significantly more males of *A. combinata* bee approaching compared to the non-manipulated negative controls (Fig 4, df = 1; $X^2 = 33.962$; $p < 0.001$). There was no difference in the number of bee approaches between the scent-manipulated *O. insectifera* plants and the *O. aymoninii*

plants (positive controls; Fig 4, df = 1; X^2 = 0.225; p = 0.635). While 21 landings and attempts for copulation occurred on *O. aymoninii* plants, we also observed 3 landings of male *A. combinata* bees on the scent-manipulated *O. insectifera* flowers (Fig 4). In one case this initiated a pseudocopulation, which may be enough for pollinia removal or pollination (Fig S1E). No landings were observed on the control *O. insectifera* plants (Fig 4). These results show the importance of the blend of palmitic acid octyl and nonyl ester as well as (*Z*)-9-C25 and (*Z*)-9-C27 alkenes as pollinator attractants in *O. aymoninii*.

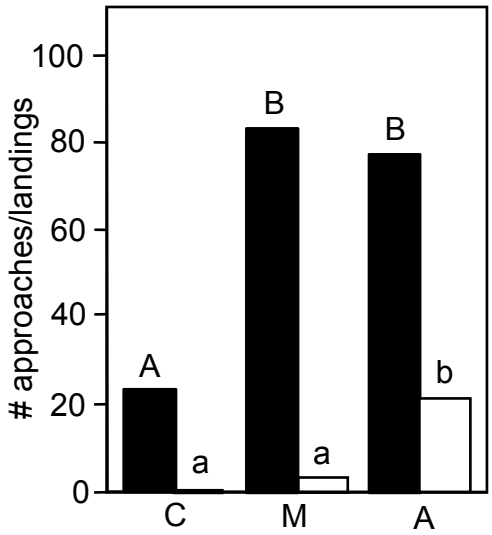

Fig. 3: Behavioral assay with scent manipulated *O. insectifera* plants and male *A. combinata* bees. Black bars represent approaches by the pollinator while white bars stand for pollinator landing on a flower. Treatments: C = control plant (non manipulated *O. insectifera*), M = manipulated *O. insectifera* plant, A = *O. aymoninii*. Different letters above the bars indicate significant differences among the treatments (X^2-test with bonferroni correction).

Density dependent selection and fecundity

In both species, plants in lower densities were found to have a tendency higher reproductive success. We found significant negative density dependent selection in the *O. insectifera* plants over all populations (Fig 4A; β = -0.177, $t_{1,141}$ = -2.13, p = 0.035). In *O. aymoninii* there was a trend towards negative density dependent selection, which was not significant (Fig 4B; β = -0.092, $t_{1,155}$ = -1.16, p = 0.249). Overall, mean fecundity (± s.d.) was found to be higher in *O. aymoninii* (0.331 ± 0.354) than in *O. insectifera* (0.106 ± 0.194) plants (Mann-Whitney U-test = 7400, p <0.001).

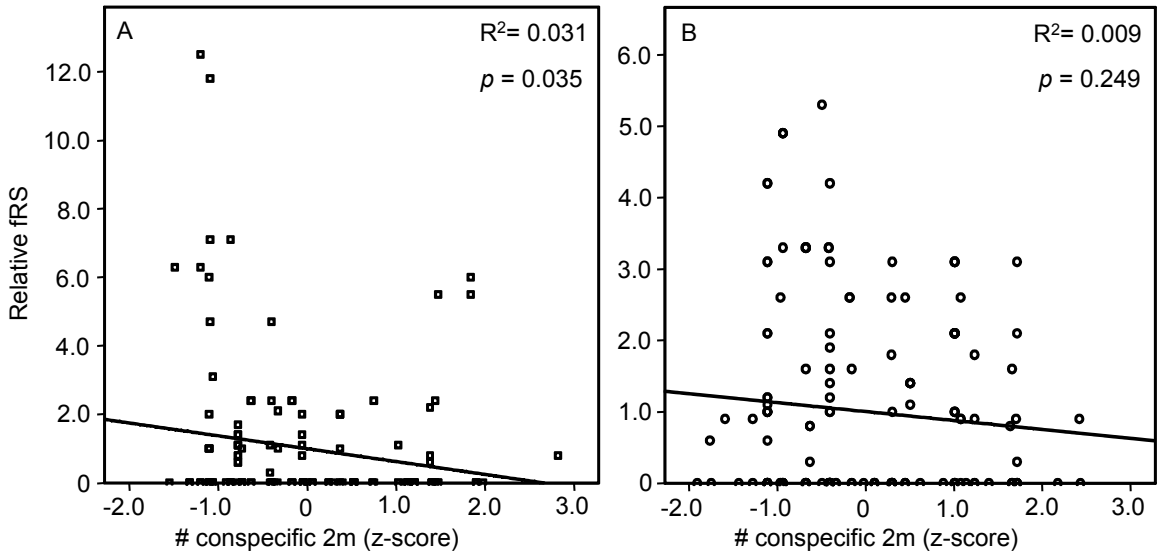

Fig. 4: Scatterplot of relative female reproductive success (Relative fRS) and z-score transformed number of conspecific within 2 m for (A) *O. insectifera* and (B) *O. aymoninii* pooled from six populations (Avey2-Avey6 and Avey8). Straight line represents the linear regression line.

Discussion

Investigations of the evolution and nature of reproductive isolation barriers, especially involving biotic interactions (e.g. pollination or mycorrhizal interactions), can provide insights into their roles in the process of speciation (Coyne and Orr 1998, 2004; Moyle et al. 2004; Scopece et al. 2007; Scopece et al. 2008; Widmer et al. 2009; Schemske 2010). In our study we experimentally measured the individual strengths of reproductive isolating barriers in two sympatric putative sister-species of the sexually deceptive orchid genus *Ophrys*. We found that reproductive isolation was mainly caused by floral isolation, more specifically by the specific attraction of pollinators, while later acting barriers were absent or very weak. Analyses of the odor bouquets show that the two species differ strongly in the relative amount of individual scent compounds, especially alkenes and ester that also played a key role in the attraction of the specific pollinator of one species. Our results show that floral isolation trough attraction of specific pollinators acts as the main barrier in reproductive isolation in sexually deceptive orchids of the *Ophrys insectifera* group while later acting barriers play an insignificant role.

Reproductive barriers in the *Ophrys insectifera* group

Prezygotic barriers have been suggested to play a more important role in preventing gene flow in plants than postzygotic barriers (Kirkpatrick and Ravigne 2002; Rieseberg and Willis 2007; Lowry et al. 2008). Indeed, studies on reproductive isolation in plants showed that prezygotic barriers are an essential factor in reproductive isolation and speciation (Bradshaw and Schemske 2003; Ramsey et al. 2003; Kay 2006; Moccia et al. 2007; Waelti et al. 2008; Xu et al. 2011). Moreover, this has been found to be especially true for specialized pollination systems as shown in sexually deceptive orchids (Schiestl and Schluter 2009; Peakall et al. 2010; Xu et al. 2011; Peakall and Whitehead 2014; Sedeek et al. 2014). Our study *of Ophrys insectifera* and *Ophrys aymoninii* also supports these findings that prezygotic barriers play a key role in reproductive isolation and speciation.

However, often prezygotic isolation is not caused by a single component but rather via a set of interacting single isolation barriers (e.g. spatial-, floral-, post-pollination prezygotic isolation) (Ramsey et al. 2003; Kay 2006; Martin and Willis 2007; Rieseberg and Willis 2007; Lowry et al. 2008). Theory suggests that single isolating mechanisms are not enough to prevent gene flow and allow coexistence of species in sympatry and thus multiple barriers must be logically assumed to exist between sympatric species (Coyne and Orr 1998, 2004). Nevertheless, there are studies in *Ophrys* supporting that floral isolation alone provides a sufficient reproductive barrier to prevent gene flow and maintain species boundaries in sympatry while later acting barriers are absent (Schiestl and Ayasse 2002; Schiestl 2005; Ayasse et al. 2011; Xu et al. 2011; Sedeek et al. 2014). Moreover, the finding of strong floral isolation acting as a major reproductive barrier is a consistent feature found in sexually deceptive orchids (Schlüter et al. 2009; Xu et al. 2012b; Peakall and Whitehead 2014; Sedeek et al. 2014). But strong floral isolation can also be found in more generalized pollination systems (Schiestl and Schluter 2009). These findings are in agreement with our study where floral isolation, trough olfactory attraction of specific pollinators, is the key reproductive barrier. Morphological differences causing a mechanical mismatch (e.g. pollinarium placement) are also regarded as an important factor in floral isolation (Schiestl and Schluter 2009; Vereecken et al. 2010). In our study we did not quantify morphological differences but as both *Ophrys* species place their pollinia on the pollinators head, we think that mechanical isolation represents a weak part of floral isolation in our study system.

Temporal isolation was shown to play, beside floral isolation, also a role in prezygotic isolation in *Ophrys* (Sedeek et al. 2014). We did not quantify phenological differences, but as both species were flowering simultaneously during our fieldwork we suggest that temporal isolation does not play an essential role as reproductive barrier. However, a recent study on the *Ophrys insectifera* group suggested that *O. aymoninii* and *O. insectifera* show ecological differences based on elevation and slope, which causes segregation of the species in sympatry (Triponez et al. 2013). Our field observations, however, showed that when the two species growing in sympatry both species typically occur within close proximity of each other (Fig S3).

Similar to other studies in *Ophrys*, later acting barriers were found to be absent, contributing nothing to reproductive isolation (Xu et al. 2011; Sedeek et al. 2014). However, nothing is known in *Ophrys* about the effects of their mycorrhizal partner on reproductive isolation. Mycorrhizal fungi that are species-specific may reduce the germination success or seedling survival of hybrids trough low fungal recruitments (Jacquemyn et al. 2011). Alternatively a geographic mosaic of fungal species may restrict the habitat preferences of their host species causing ecological segregation but our field observations showed that both species can grow side by side when occurring in sympatry (Fig S3). Moreover, it has been suggested that mycorrhizal associations contribute little to reproductive isolation in closely related *Orchis* species occurring in sympatry (Jacquemyn et al. 2011). This was found to be in agreement to our results. When occurring in sympatry we did not find that the individual *Ophrys* species were associated with species-specific mycorrhizal partners but rather share the mycorrhizal fungi, with a marked, shared preference for one Tulasnellaceae species. On the one hand, this family is common on *Ophrys* species (Jacquemyn et al. 2015; Pecoraro et al. 2015). On the other hand, the sharing of similar fungi in sympatric orchid taxa is thought to be unlikely, because sympatric species are assumed to avoid fungal sharing (Jacquemyn et al. 2014; Jacquemyn et al. 2015). The result suggests that specificity of mycorrhizal symbiosis is unlikely to contribute to reproductive isolation.

All this supports our findings that floral isolation, more specifically the attraction of specific pollinators trough floral volatiles, acts as key barrier in reproductive isolation. Given that we also only found only one type of barrier in this system, it is very likely that adaptation to different pollinators played a key role in the initial steps of speciation in this group of *Ophrys*.

Floral scent as key reproductive barrier

Pollinator attraction and specificity in *Ophrys* appears to be mainly caused by floral odors emitted by the plants (Agren and Borg-Karlson 1984; Schiestl et al. 1999, 2000; Mant et al. 2005a; Ayasse 2007; Ayasse et al. 2011; Xu et al. 2011; Sedeek et al. 2014). It has been reported that in *Ophrys* pollinated by male solitary bees specific alkanes and alkenes (cuticular hydrocarbons) attract male bees and stimulate mating (Schiestl et al. 2000; Mant et al. 2005a; Vereecken et al. 2007; Xu et al. 2012a). This would explain the high levels of alkenes in *O. aymoninii* (especially (*Z*)-9-penta-and heptacosene), which is pollinated by male bees while *O. insectifera* is pollinated by male digger wasps. Our finding of high levels of esters in *O. aymoninii* is in agreement to (Borg-Karlson et al. 1993) who also detected higher amounts of esters in *O. aymoninii* plants compared to *O. insectifera*. Altough, high levels of esters have also been found in *Ophrys garganica*, nothing is known about their function and if they act as sex pheromone (Sedeek et al. 2014). However, a study in *Ophrys sphegodes* and its pollinator, male *Andrena nigroaenea* bees, showed that esters elicited electrophysiological responses in the bees (Ayasse et al. 2000), which was also in agreement to our study where esters were found to be electrophysiological active in male *Andrena combinata* bees. Unfortunately, we were not able to obtain male digger wasps to perform GC-EAD analysis on *O. insectifera* and determine key compounds or perform behavioral assays. The key attractants in *O. insectifera* therefore still remain unknown. Nevertheless, our findings shows that floral scent, especially alkenes and esters, plays a key role in floral isolation in the *Ophrys insectifera* group and could act a sole reproductive barrier.

Evidence for pollinator-driven sympatric speciation?

Sympatric speciation, strictly described as "the emergence of a new species from a population where mating is random with respect to the birthplace of the mating partners"(Gavrilets 2003), is one of the most contested theory in evolutionary biology. An exception is the well understood process of polyploidization, which gives many clear-cut examples of sympatric speciation and is relatively common in plants (Coyne and Orr 2004; Rieseberg and Willis 2007). Although common in plants, polyploidization can be excluded in our study system as a driver of sympatric speciation given that the two

investigated *Ophrys* species have the same ploidy level. The major obstacle in homoploid sympatric speciation is that mating and recombination in a population will easily break down associations between alleles contributing to reproductive isolation and therefore preventing the formation of distinct groups. However in the last two decades, the theory has experienced a revival and new models as well as new empirical studies emerged supporting homoploid sympatric speciation so that nowadays sympatric speciation is seen as theoretically possible even by critical minds (Schliewen et al. 1994; Dieckmann and Doebeli 1999; Coyne and Orr 2004; Barluenga et al. 2006; Savolainen et al. 2006).

The classical model for pollinator-driven speciation states that plants growing in habitats with different pollinator-environments will adapt to their local most effective pollinators (Grant and Grant 1965; Stebbins 1970; Johnson 2006). Such adaptations to different pollinators can cause floral isolation and result in reproductive isolation preventing gene flow (Johnson 2010; Schiestl 2012). Alternatively, adaptation to a new pollinator may be driven by low fecundity in a deceptive system where pollinators learn to avoid deceptive plants (Paulus and Gack 1990; Ferdy et al. 1998; Wong and Schiestl 2002; Tremblay et al. 2005; Xu et al. 2011). Such a situation may lead to negative density dependent selection, favoring the switching of pollinators in sympatry (Waser and Campbell 2004; Xu et al. 2011). This situation may be true for *O. insectifera* where plants showed very low fecundity and have been found to be under significant negative density dependent selection, theoretically favoring a switch of pollinators even in sympatry. Our behavioral assay strikingly demonstrated how small changes in the relative scent composition of *O. insectifera*, depicting a mutation, might already attract *Andrena combinata* bees. But it is not clear if this also leads to a lower attraction to the original pollinator of O. insectifera, which is important if gene flow should cease. Nevertheless, sexually deceptive orchids have been clearly demonstrated to have highly specific plant-pollinator relations where attraction of a new pollinator in sympatry may indeed cause floral isolation and ultimately speciation (Schlüter et al. 2009; Peakall et al. 2010; Ayasse et al. 2011).

However, the attraction of a new pollinator demands selection for changes in floral traits at the genetic level and these traits should have a simple genetic basis for speciation to occur in sympatry. It is generally assumed that changes in floral traits are quite commonly based on several genes with minor effect (Schemske and Bradshaw 1999; Martin et al. 2007; Moyle 2007). However, studies in *Mimulus* and *Petunia* showed that single genes can have major effects on certain flower traits and pollinator specificity

(Bradshaw and Schemske 2003; Hoballah et al. 2007). More importantly, it has been shown in *Ophrys* that changes in floral odors, can be caused by only few genes responsible for the biosynthesis of the floral odor compounds (Schlüter et al. 2011; Xu et al. 2012a; Xu and Schlüter 2014; Sedeek et al. 2016).

To support a sympatric speciation event in our study system, it is crucial to know the phylogenetic relation between the two species. While both species are in a basal monophyletic group (together with *O. subinsectifera*) within the genus *Ophrys*, it has not been yet clearly shown if they are also sister-species (Breitkopf et al. 2015). Nevertheless, given the sympatric occurrences of an endemic species with the widespread species, as well as floral isolation as the sole reproductive barrier discovered so far, *O. insectifera* and *O. aymoninii* may represent a promising candidate for investigating sympatric speciation.

Conclusion

The further analysis of the chemical ecology in the pollinators of our system will help us gain better insights in the function and relevance of individual floral scent compounds, especially about the esters in *O. aymoninii*. Our study system may also represent an interesting candidate for studying sympatric speciation. However, for proving sympatric speciation it is crucial to show that both species are sister-species and therefore a fine resolved phylogeny is needed. Moreover, it would be essential to show that the local *O. insectifera* plants are closer related to *O. aymoninii* then to *O. insectifera* populations in other regions. Finally, identification of the genetic background of the traits responsible for reproductive isolation in the *Ophrys insectifera* group will help us understand the mechanisms of speciation in these plants.

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Supplementary data

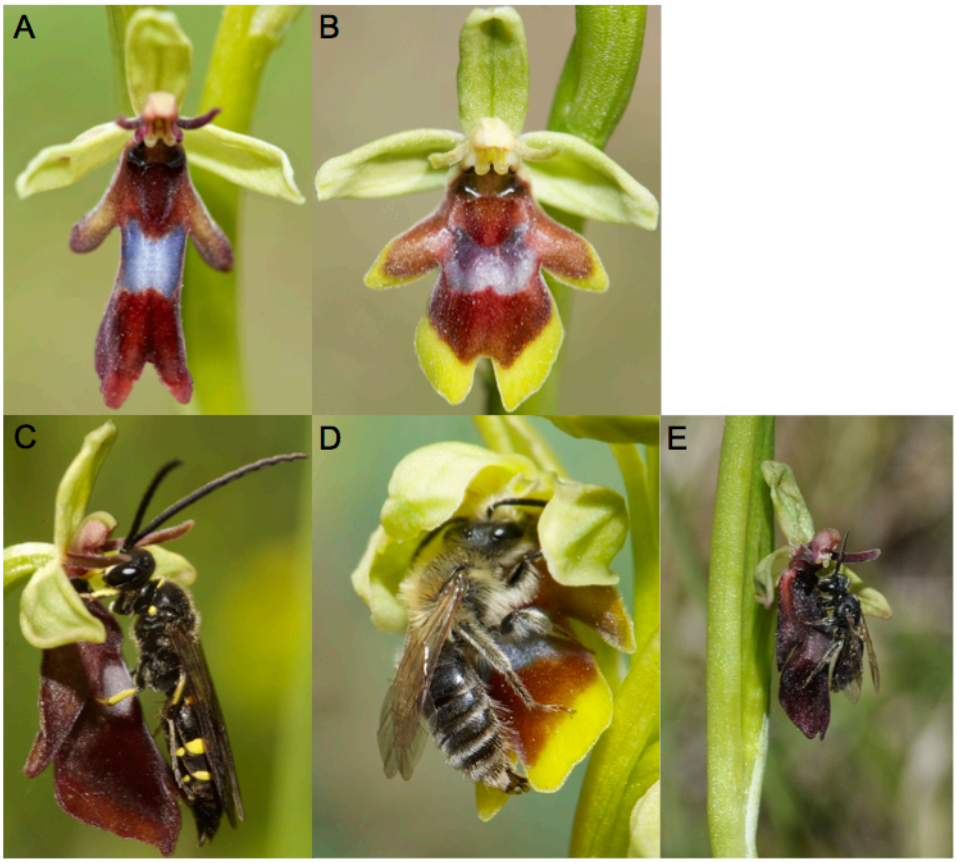

Fig. 1: A) *Ophrys insectifera* (Daniel Gervasi). B) *Ophrys aymoninii* (Daniel Gervasi). C) *O. insectifera* with male *Argogorytes mystaceus* (Nicolas Vereecken). D) *O. aymoninii* with male *Andrena combinata* (Nicolas Vereecken) E) Scent manipulated *O. insectifera* with male *A. combinata* (Daniel Gervasi)

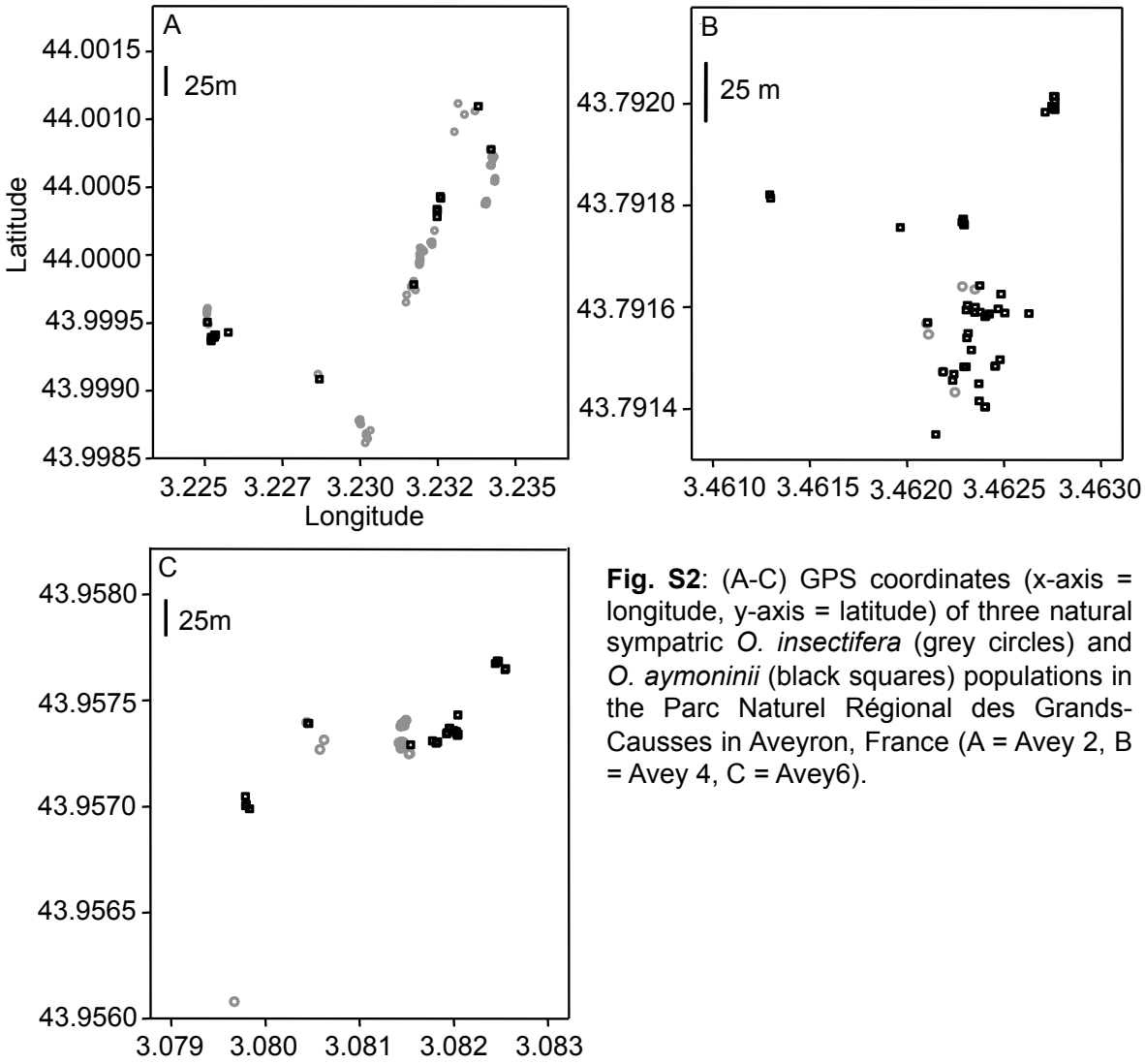

Fig. S2: (A-C) GPS coordinates (x-axis = longitude, y-axis = latitude) of three natural sympatric *O. insectifera* (grey circles) and *O. aymoninii* (black squares) populations in the Parc Naturel Régional des Grands-Causses in Aveyron, France (A = Avey 2, B = Avey 4, C = Avey6).

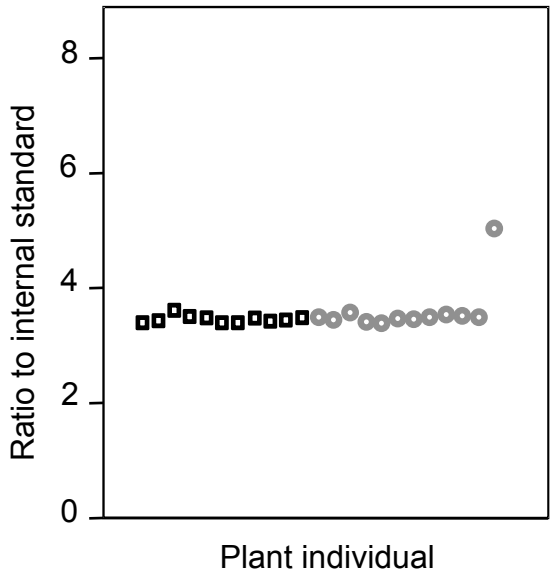

Fig. S3: Ploidy levels of pollinia from *O. insectifera* and *O. aymoninii plants*. Each data point represents the relative ratio between pollinia and internal standard from an individual plant (black squares= *O. aymoninii*, grey circles= *O. insectifera*).

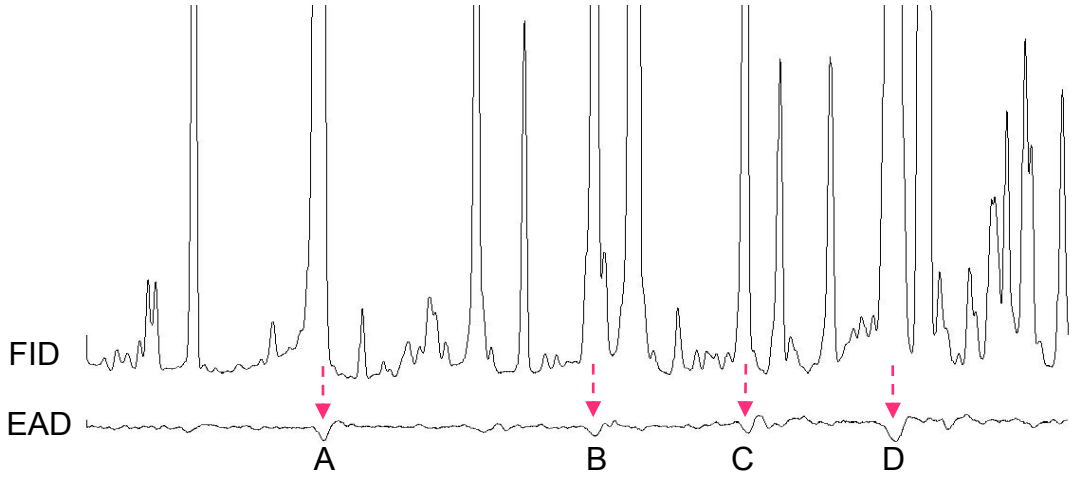

Fig. S4: Gas chromatographic analyses with electroantennographic detection (GC-EAD) of *Ophrys aymoninii* labellum extract, using antennae of a male *Andrena combinata* bees which are pollinator of *O. aymoninii.* Pollinators respond to four peaks identified as A = tricosane , B = (*Z*)-9-pentacosene, C = palmitic acid octyl ester, D = palmitic acid nonyl ester/ (*Z*)-9-heptacosene. FID, flame-ionization detector.

Table S1: Coordinates of all 7 populations with description of the surroundings

Population	Species	Coordinates	Description
Avey 2	*O. aymoninii*	44° 1' 40" N, 3° 15' 52" E	Dense pine forest with open grass patches
Avey 3	*O. aymoninii* *O. insectifera*	43° 59' 56" N, 3° 13' 39" E	Dense pine forest and open grass fields with single pines
Avey 4	*O. aymoninii* *O. insectifera*	43° 47' 29" N, 3° 27' 43" E	Dense pine forest and open grass fields with single pines
Avey 5	*O. insectifera*	43° 59' 30" N, 2° 56' 41" E	Free grass field bordered by oak forest and wheat field
Avey 5_1	*O. insectifera*	44° 0' 18" N, 2° 57' 36" E	Pine forest with abandoned vineyard
Avey 6	*O. aymoninii* *O. insectifera*	43° 57' 26" N, 3° 4' 52" E	Dense pine forest and open grass fields with single pines
Avey 8	*O. insectifera*	43° 54' 33" N, 3° 17' 41" E	Dense pine forest

Table S2: The 19 fungal OTUs found in *O. insectifera* and *O. aymoninii*, with number of sequences (Seq.) amplified among the 396 mycorrhizal samples and number of individual orchids (Ind.) where the OUT was found (out of, respectively, 26 and 24 sampled individuals).

	GB accession number(s)	**Putative taxonomic affiliation**	***O. aymoninii***		***O. insectifera***	
			Seq.	Ind.	Seq.	Ind.
Orchid mycorrhizal fungi			91	27	73	30
T1	KF871201	*Tulasnella*	78	23	60	21
T2	KF871202	*Tulasnella*	3	1	1	1
T3	KF871203	*Tulasnella*	0	0	1	1
S1	KF871204	Sebacinales cl. B	2	1	11	7
S2	KF871205	Sebacinales cl. B	8	2	0	0
Endophytic fungi			8	7	6	6
E1	KF871206	*Leptodontidium sp.*	0	0	3	3
E2	KF871207	*Tetracladium sp.*	2	2	1	1
E3	KF871208	*Tetracladium sp.*	1	1	0	0
E4	KF871209	*Tetracladium sp.*	0	0	1	1
E5	KF871210	*Neonectria sp.*	1	1	0	0
E6	KF871211	*Gymnopus sp.*	2	1	0	0
E7	KF871212	Helotiales sp.	0	0	1	1
E8	KF871213	*Ceratobasidium sp.*	1	1	0	0
E9	KF871214	*Cladosporium sp.*	1	1	0	0
Ectomycorrhizal fungi			8	5	1	1
M1	KF871215	*Rhizopogon sp.*	5	2	0	0
M2	KF871216	*Tricholoma sp.*	1	1	0	0
M3	KF871217	*Russula sp.*	1	1	0	0
M4	KF871218	Aethaliales sp.	1	1	0	0
M5	KF871219	Thelephoraceae sp.	0	0	1	1

FINAL REMARKS

The study of the mechanisms that generated todays astonishing floral diversity is one the key aspects in evolutionary biology. In this thesis the focus was set on pollinator-driven mechanisms in generalized and specialized plant systems as a potential driver of floral diversity and plant speciation. More specifically, emphasis was laid upon pollinator-mediated selection and floral isolation. This thesis shows how different pollinators can strongly influence floral evolution (but also each other) and can act as strong reproductive barriers between plants in sympatry. Thus the results support the common view that pollinators play an important role in the evolution of floral signals as well as plant speciation.

Trough an experimental evolution approach in generalized plants, this thesis could show that in spatially separated pollinator environments divergent selection acts as a major driver of floral divergence (Chapter I). This implies that adaptations to different pollinators are most likely a key driver for floral diversity. It furthermore shows the potential consequence that shifts in pollinator environments can have not only on floral evolution but also on the mating-systems in plants as pollen limitation was found to be a promoter of selfing. But Chapter II implies that in a mixed pollinator environment, the importance of pollinators as driver of floral diversity may be much lower. In particular, it shows that a highly efficient pollinator does not necessarily dictate selection based on its preferences and that a low efficient pollinator may strongly influence the selection imposed on the floral traits.

Generalized plants exposed to different pollinators have shown to undergo phenotypic changes with time, but these changes were not sufficient for the emergence of floral isolation (Chapter 1). The results further indicate that prepollination barriers do not evolve prior to postzygotic ones. Therefore, I argue that in generalized plants systems floral isolation may play a rather minor role in reproductive isolation. It would have been surprisingly if strong floral isolation would have emerged after only nine generation, specifically in such a generalized plant with a conservative floral *Bauplan* as *Brassica rapa*. However, given enough time floral isolation could become more prevalent as bumblebees already show to have a first-choice preference for “their” plants. Contrary to the generalized system, floral isolation seems to be the major (if not only one) reproductive barriers between two highly specialized sexually deceptive orchids in

sympatry (Chapter III). Moreover, in this highly specialized pollination system floral divergences between the two species are most likely a consequence of adaptations to different pollinators, which could have occurred in sympatry. I argue that in such highly specialized pollination systems the pollinators not only act as a major driver of floral diversity but also of plant speciation which can occur in sympatry.

Together, I conclude that adaptations of plants to different pollinators play an essential role in evolution of the floral diversity and plant speciation. But my thesis also highlights that floral evolution as well as reproductive isolation can be strongly influenced by the pollination system as well by the way the pollinators may interact with each other. Of interest is the prevalent role floral scent plays in these specialized and generalized plant systems. The study of floral scent in such a scale has never been done so far and gives us a deep insight in how floral scent can be shaped by pollinators (Chapter I) and can be a central aspect in reproductive isolation (Chapter III).

However, this thesis offers not only answers but also generates a multitude of novel questions and research goals. A major aspect, which has been not touched in my thesis, is the study of the genetic basis of the floral traits that have undergone divergent evolution or contribute to floral isolation. Furthermore, it is crucial to study the pollinator sensory system and innate preferences to understand why certain floral signals were under selection and others not. Often plant-pollinator studies focus mainly on the plant while the pollinator ecology and biology are rarely known. Additionally, while it is difficult to quantify, it is essential to include male reproductive success into further selection analysis. This is even more important in experimental evolution studies over several generations if one wants to link pollinator-mediated selection and floral evolution.

Ultimately, this thesis also shows how strong experimental evolution can be in studying the mechanisms of pollinator-driven floral evolution. While studies in the field offer us the real natural conditions it is often impossible to detect and incorporate all the factors influencing floral evolution especially over multiple generations. I think here lays the strength of the experimental evolution approach as it allows one to specifically focus on the effects that pollinators have on floral signals without other external factors. In my view, more experimental evolution studies in plant-pollinator interactions will help us significantly to understand the process of plant adaptation to pollinators. Additionally, the combination with variations in abiotic conditions or the addition of herbivores may also give excellent insights on how these factors interact and their importance in floral

evolution. However, factors limiting the study of plant-pollinator interactions with experimental evolution can be found in the generation time of plants as well as availability of suitable pollinators. Plants with long generation time will hardly be suited for a multigenerational study as well as when floral signals show low variability upon which selection may act. On the other side the availability of pollinators all around the year is rarely met reducing the variety of available pollinators, especially in plants with a less generalized pollination system. Nevertheless, while there are risks in experimental evolution studies (but risks exist everywhere) it also offers great rewards. I hope to have sparked a fire in future researchers for studying the exceptional floral diversity trough experimental evolution studies.

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